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Investigating the molecular basis of AMKL and MDS

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Abstract

Malignant haematopoiesis is usually associated with various genetic lesions such as chromosomal translocations or mutations in individual genes. This thesis investigates the genetic basis of two such syndromes, acute megakaryoblastic leukaemia (AMKL) and myelodysplastic syndrome (MDS).

The most common constitutional aneuploidy with predisposition to leukaemia is trisomy 21, also known as Down Syndrome (DS). DS children have a 500-fold increased risk for AMKL. Somatic mutations acquired during foetal haematopoiesis in the GATA1 transcription factor are detected in megakaryoblasts from all the DS patients with AMKL. Here we show that the *Gata1* mutation co-operates with the chromosome 21 gene, *ERG-3,* to immortalize foetal megakaryocyte progenitors with the phenotype of AMKL megakaryoblasts. We show that ERG-3 promotes megakaryopoiesis and acts as an oncogene and that progenitor cells harbouring a *Gata1* mutation plus ERG-3 or ERG-3 alone lead to rapid development of leukaemia *in vivo*. Our data support a model where trisomy 21 overexpressed genes, that promote foetal megakaryopoiesis, co-operate with mutations that arrest development and lead to DS-AMKL. This is also the first direct demonstration of the leukaemogenic activity of full length ERG protein, possibly explaining the poor prognosis of the acute myeloid leukaemias with high expression of ERG.

DS patients are also predisposed to TMD (transient myeloproliferative disorder) and MDS (myelodysplastic syndrome) prior to AMKL development. Myelodysplastic syndromes are a group of clonal haematopoietic disorders,

characterised by aberrant proliferation and differentiation of cells of the myeloid lineage resulting in severe cytopenia and dysplasia of myeloid, erythroid and megakaryocytes. The most common chromosomal translocation associated with MDS is the t(3;5)(q25q35) translocation. This rearrangement results in a fusion transcript comprised of nucleophosmin (*NPM*) gene and myeloid leukaemia factor 1 (*MLF1*) gene. To determine the importance of the NPM-MLF1 fusion protein in the development and progression of MDS to AML, its role in myelopoiesis and megakaryopoiesis was investigated. Our results show that NPM-MLF1 affects the differentiation of myeloid cells. Our preliminary data predicts that NPM may have a role in megakaryopoiesis and its interaction with NPM-MLF1 may affect the function of the fusion protein.

Declaration

I, Samira Salek-Ardakani, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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This thesis is dedicated to my mum and dad

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Abbreviations

2-Me	2-mercaptoethanol
5-FU	5-Fluorouracil
AD	Activation Domain
AGM	Aorta Gonad Mesonephros
ALK	Anaplastic Lymphoma Kinase
ALL	Acute Lymphoblastic Leukaemia
AMKL	Acute Megakaryoblastic Leukaemia
AML	Acute Myeloid Leukaemia
AML1	Acute Myeloid Leukaemia 1
APC	Allophycocyanin
APP	Amyloid beta Precursor Protein
APS	Ammonium Persulphate
ARF	Alternative Reading Frame
BACH1	Basic leucine zipper transcription factor 1
BCR-ABL	B Cell Receptor-Abelson Tyrosin Kinase
BFU-E	Burst Forming Unit Erythroid
BM	Bone Marrow
BSA	Bovine Serum Albumin
CAPS	N-Cyclohexyl-3-aminopropanesulfonic acid
CBP	CREB Binding Protein
CBS	Cystathionine β-Synthase
CF	C-terminal zinc Finger
CFU	Colony Forming Unit

CFU-GEMM	Colony Forming Unit Granulocyte, Erythrocyte, Macrophage and Megakaryocyte
CFU-MK	Colony Forming Unit Megakaryocyte
CGI	Congenital Gastrointestinal tract
CHD	Congenital Heart Disease
CLP	Common Lymphoid Progenitor
CML	Chronic Myeloid Leukaemia
CMML	Chronic Myelomonocytic Leukemias
CMP	Common Myeloid Progenitor
CNC	Cap-N-Collar
CNS3-COP1	Central Nervous system Stage 3- Constitutively Photomorphogenic 1
DMEM	Dulbeccos Modified Eagle Media
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic Acid
DNA-PK	DNA-Protein Kinase
DREF	DNA Replication-related Element (DRE)-binding Factor
DS	Down Syndrome
DSCR	Down Syndrome Critical Region
DYRK1A	Dual-specificity tyrosine-Regulated Kinase 1A
DTT	Dithiothreitol
E	Embryonic Day
EB	Embryoid Body
EDTA	Ethylene-Diamine-Tetra-Acetic acid
EPO	Erythropoietin

ERG	ETS-Related Gene
ERS	ERG Specfic domain
ES	Embryonic Stem cell
ETO	Eight-Twenty-One
ETS	E26 (E Twenty Six) avian erythroblastosis virus
ETV6	Ets Translocation Variant gene 6
EVI1	Ecotropic Viral Integration site 1
FA	Fanconi Anaemia
FAB	French-American-British association
FCS	Foetal Calf Serum
FITC	Fluorecein Isothiocyanate
FL	Foetal Liver
FLI1	Friend Leukemia virus Integration 1
FLT3	FMS-Like Tyrosine kinase 3
FOG1	Friend of GATA1
GATA1	GATA binding protein 1
GATA1s	GATA1 short
G-CSF	Granulocyte-Colony Stimulating Factor
GFP	Green Fluorescent Protein
GMP	Granulocyte-Monocyte Progenitor
GP	Glycoprotein
HBS	Hepes Buffered Saline
HDM2	Human Double Minute2
HCl	Hydrochloric acid
HIV	Human Immunodeficiency Virus

HLS-7	Haematopoietic Lineage Switch-7
hnRNP-U	Heterogeneous Nuclear Ribonucleoprotein U
HSC	Haematopoietic Stem Cell
Ig	Immunoglobin
IL	Interleukin
INT	Iodophenyl Nitrophenyl Tetrazolium
IRES	Internal Ribosome Entry Site
IRF	Interferon Regulatory Factor
ITD	Internal Tandem Duplication
JAK	Janus Kinase
kb	Kilobases
kDa	Kilodalton
LMPP	Lymphoid primed Multi-Potent Progenitor
LOH	Loss Of Heterozygosity
LSC	Leukaemic Stem Cell
LT-HSC	Long Term-Haematopoietic Stem Cell
LTR	Long Term Repeat
MI	Meiotic Division I
MII	Meiotic Division II
MACS	Magnetic Activated Cell Sorter
MAF	Musculoaponeurotic Fibrosarcoma oncogene
MCS	Multiple Cloning Site
M-CSF	Macrophage-Colony Stimulating Factor
MDS	Myelodysplastic Syndrome
MEP	Megakaryocyte-Erythroid Progenitor

MGDF	Megakaryocyte Growth and Development Factor
MHC	Major Histocompatibility Complex
MKL	Megakaryoblastic leukaemia
MLF1	Myeloid Leukaemia Factor 1
MLL-ENL	Mixed Lineage Leukaemia-Eleven-Nineteen Leukaemia
MOZ-TIF2	Monocytic leukaemia zinc finger protein-Transcriptional Intermediary Factor 2
MPP	Multipotent Progenitor
MSC	Mesenchymal Stem Cell
MSCV	Murine Stem Cell Virus
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2- (4-sulfophenyl)-2H-tetrazolium
MX1	Interferon induced GTP binding protein
NaCl	Sodium chloride
NaOAc	Sodium Acetate
Neo^r	Neomycin resistance
NES	Nuclear Export Signal
NF	N-teminal zinc Finger
NF-E2	Nuclear Factor-Erythroid 2
NLS	Nuclear Localisation Signal
NOD	Non-Obese Diabetic
NPM	Nucleophosmin
PAGE	PolyAcrylamide Gel Electrophoresis
PB	Peripheral Blood
PBS	Phosphate Buffered Saline

PE	Phycoerthyrin
PerCP	Peridinin Chlorophyll Protein
PI	Propidium Iodide
PLZF	Promyelocytic Leukaemia Zinc Finger protein
PML-RARα	Promyelocytic – Retenioic Acid Receptor alpha
PNT	Pointed Domain
PTS	Paris-Trousseau Syndrome
RA	Refractory Anaemia
RAEB	Refractory Anaemia with Excess Blasts
RARS	Refractory Anaemia with Ringed sideroblasts
Rb	Retinoblastoma
RBM15	RNA Binding Motif protein 15
RIPA	RadioImmunoPrecipitation Assay
RNA	Ribonucleic Acid
RPMI	Roswell Park Memorial Institute
RT	Room Temperature
RUNX1	Runt related transcription factor 1
SCF	Stem Cell Factor
SCID	Severe Combined Immunodeficiency
SD	Standard Deviation
SDS	Sodium Dodecyl-Sulphate
SOD1	Superoxide Dimutase1
SSC	Sodium Chloride Sodium Citrate
STAT	Signal Transduction and Activator of Transcription
ST-HSC	Short Term Haematopoietic Stem Cell

TC	Transchromosomic
TCR	T Cell Receptor
TEL	Translocation-Ets-Leukaemia
TEMED	N,N,N',N'-Tetramethylethylenediamine
TL	Transient Leukaemia
TMD	Transient Myeloproliferative Disorder
TPO	Thrombopoietin
Tris	Tris (hydroxylmethyl)methylamine
UV	Ultraviolet
WT	Wild Type
YS	Yolk Sac
ZN	Zinc Finger

Publication

The work described in Chapters 3 and 4 forms part of a manuscript, which has been submitted for publication.

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Chapter 1

Introduction

Chapter 1: Introduction

Human leukaemias and haematopoietic disorders arise primarily as a result of abnormal regulation and development of stem and progenitor cells that give rise to red and white blood cells. Overexpression of a gene, genetic mutations, chromosomal aneuploidy or translocations can result in the aberrant regulation of cellular proliferation and differentiation that can lead to proliferative disorders and leukaemic development.

Down Syndrome (DS) is an example of a disorder that involves chromosomal aneuploidy (trisomy 21). Down syndrome neonates are highly predisposed to development of acute megakaryoblastic leukaemia (AMKL). DS-AMKL is almost always coupled with a mutation in the *GATA1* gene, which is crucial for the development and differentiation of platelet producing cells, megakaryocytes. In this study we examined the potential collaboration between *ERG-3*, a gene localised to chromosome 21, and *GATA1* mutation in leukaemogenesis. Below, current models of mouse haematopoiesis, different DS murine models and the transcriptional regulation of megakaryopoiesis, will be discussed. The function of *GATA1* mutation in haematopoiesis and disease will also be addressed.

Individuals with DS are also predisposed to a myeloproliferative disease, myelodysplastic syndrome (MDS), prior to AMKL development. In addition, trisomy 21 has been detected as a sole genetic abnormality in some non-DS AML as well as patients suffering from MDS alone (Wan *et al.*, 1999). MDS is associated with poor prognosis and there are number of genetic mutations and chromosomal

translocations correlated with MDS. The second part of this study was to elucidate the functional importance of the *NPM-MLF1* fusion gene created as a result of t(3;5) associated with MDS. The clinical characteristics of MDS and the role of wild type NPM. MLF1 and NPM-MLF1 will be discussed below.

1.1 Haematopoietic development in mice

Haematopoiesis refers to the formation and the development of red and white blood cells which occurs during embryogenesis and throughout normal life. Mature blood cells of all lineages are derived from pluripotential long-term (LT) haematopoietic stem cells (HSCs) which are a rare, quiescent population of cells representing less than 0.1% of total nucleated cells in the bone marrow. These cells can reconstitute the haematopoietic system of lethally irradiated recipient mice and the long-term self-renewal potential of these cells has been demonstrated by the serial transplantation of HSCs into secondary and tertiary recipient mice (Smith *et al.*, 1991; Spangrude *et al.*, 1995). LT-HSCs give rise to short-term (ST) HSCs that have limited self-renewal capacity of around three months and these cells can generate multipotent progenitors (MPP) that do not self renew but can diverge and commit into two main pathways of the haematopoietic system, the myeloid and the lymphoid lineage (Christensen and Weissman, 2001). The LT-HSCs, ST-HSCs and MPPs exist in a small population of cells in the bone marrow that lack the expression of lineage marker (Lin) and express stem cell associated markers Sca1 and c-Kit ($Lin^-Sca1^+c\text{-}kit^{high}$ or LSK). These can further be distinguished by the expression of CD34 and the cytokine receptor FMS-like tyrosine kinase 3 (FLT3). LT-HSCs lack the expression of both CD34 and FLT3 (Osawa *et al.*, 1996;

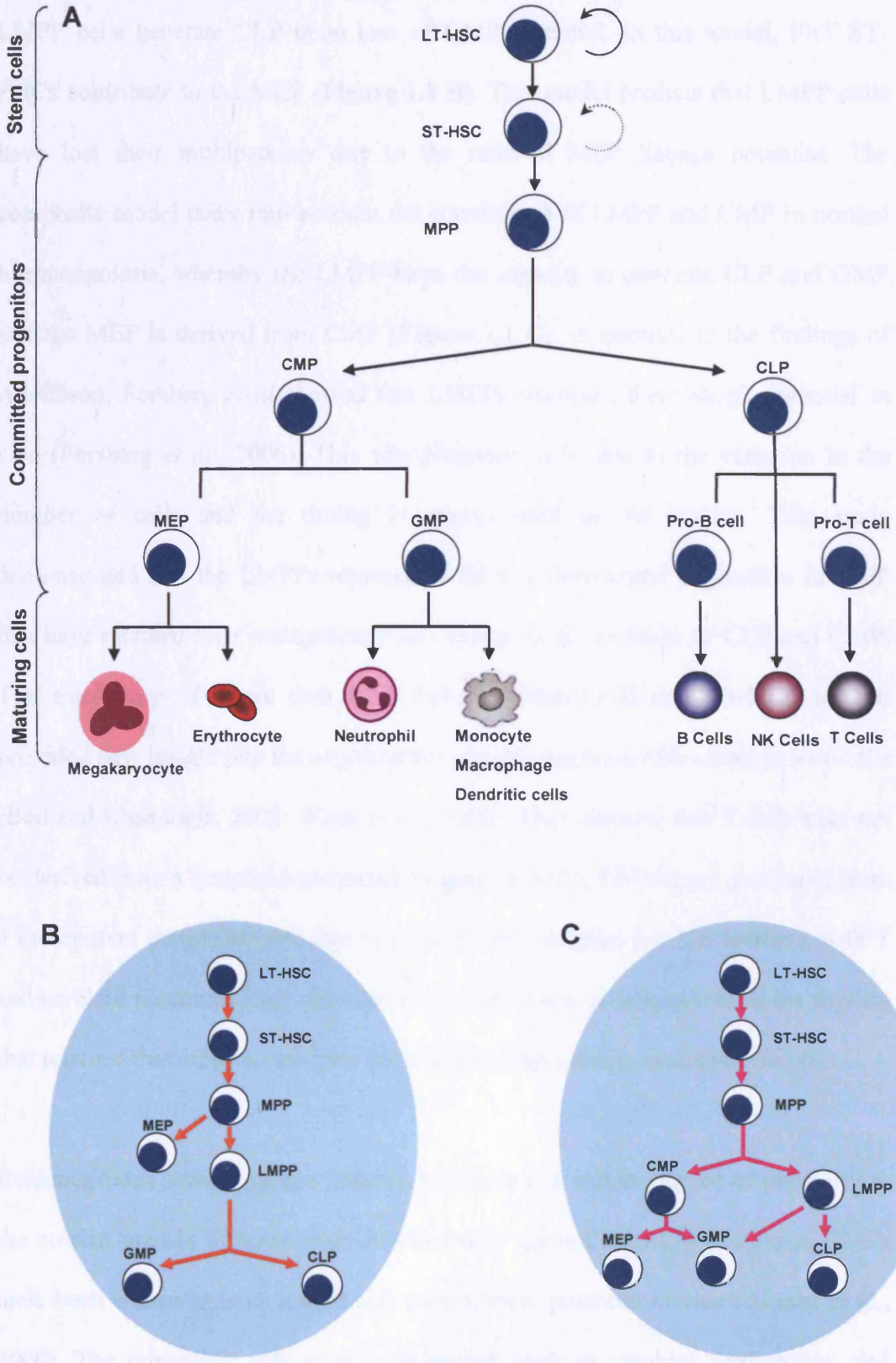
Adolfsson *et al.*, 2001) whereas ST-HSCs are $CD34^{+}FLT3^{-}$ (Yang *et al.*, 2005).

1.1.1 Models of haematopoiesis

The conventional model of haematopoiesis involves the commitment and differentiation of ST-HSCs or MPPs along one of two pathways, giving rise to either the common myeloid progenitor (CMP) or to the common lymphoid progenitor (CLP) (Ogawa *et al.*, 1993; Morrison *et al.*, 1995; Akashi *et al.*, 2000). CMPs are multi-potent cells that can differentiate into either granulocyte-macrophage progenitor (GMP) or megakaryocyte-erythrocyte (MegE) progenitor cells. The GMP can give rise to granulocytes (neutrophils, basophils and eosinophils) and monocytes whereas the MEP can generate erythrocytes or platelet producing megakaryocytes (**Figure 1.1 A**). The CLP have the capacity to develop into pro-B and pro-T cell progenitors that generate B lymphocytes and T lymphocytes respectively. Natural Killer cells are also derived from CLPs.

Improvements in the isolation of lineage specific progenitors have lead to the proposal of more flexible hierarchical models of haematopoiesis. An alternative model was suggested by Adolfsson *et al* following the identification of another LSK progenitor cell, the lymphoid primed multipotent progenitor (LMPP), which is $LSKCD34^{+}Flt3^{+}$ (Adolfsson et al., 2001; Adolfsson *et al.*, 2005). LMPP can generate GMP but in contrast to ST-HSCs have lost the ability to commit to the MK and E lineage as shown by the down regulation of erythroid and

Figure 1.1. Models of haematopoiesis. A. Conventional model. Haematopoietic stem cells (HSCs) can be divided into long term (LT) HSCS capable of indefinite self renewal which can provide life long maintenance of the entire haematopoietic system or short term (ST) HSCs that can self renew for a limited period. ST-HSCs can give rise to multipotent progenitor (MPP) cells and can self renew and commit to differentiate into either the common lymphoid progenitor (CLP) or common myeloid progenitor (CMP). CLP gives rise to T and B lymphocytes as well as natural killer (NK) cells, whereas CMP gives rise to granulocyte-monocyte progenitor (GMP) or megakaryocyte-erythrocyte progenitor (MEP). GMPs produce granulocytes (basophils, neutrophils and eosinophils) and monocytes. MEPs develop into either megakaryocytes or erythrocytes. **B. Alternative model.** In this model MPPs develop into a lymphoid primed multipotent progenitor (LMPP) following the loss of megakaryocyte and erythroid potential. LMPP can generate GMP and CLP. **C. Composite model.** Coexistence of LMMP and CMP where LMPP can give rise to CLP as well as GMP. (Adapted from Adolfsson *et al.*, 2005).


A
Stem cells
Committed progenitors
Maturing cells
LT-HSC
ST-HSC
MPP
CMP
CLP
MEP
GMP
Pro-B cell
Pro-T cell
Megakaryocyte
Erythrocyte
Neutrophil
Monocyte
Macrophage
Dendritic cells
B Cells
NK Cells
T Cells
B
LT-HSC
ST-HSC
MPP
MEP
LMPP
GMP
CLP
C
LT-HSC
ST-HSC
MPP
CMP
LMPP
MEP
GMP
CLP

megakaryocytic specific transcription factors in the LSKCD34^{+}Flt3^{+} population. LMPP cells generate CLP upon loss of GMP potential. In this model, Flt3^{-} ST-HSCs contribute to the MEP (**Figure 1.1 B**). This model predicts that LMPP cells have lost their multipotency due to the reduced MEP lineage potential. The composite model takes into account the coexistence of LMPP and CMP in normal haematopoiesis, whereby the LMPP have the capacity to generate CLP and GMP and the MEP is derived from CMP (**Figure 1.1 C**). In contrast to the findings of Adolfsson, Forsberg *et al* showed that LMPPs sustained their MegE potential *in vivo* (Forsberg *et al.*, 2006). This was proposed to be due to the variation in the number of cells and the timing of assays used in the studies. This study demonstrated that the LMPPs represent a more differentiated population of MPP that have retained their multipotency and appear to be upstream of CLP and CMP. The emergence of recent data from Bell and Bhandoola and Wada *et al*, has provided new insight into the organization and development of haematopoietic cells (Bell and Bhandoola, 2008; Wada *et al.*, 2008). They propose that T cells may not be derived from a lymphoid-restricted progenitor cell (CLP) but are generated from a multipotent progenitor cell that has lost B cell potential but has retained both T and myeloid potential. They demonstrate the existence of progenitors in the thymus that retained the ability to generate both macrophages and granulocytes *in vivo*.

Evidence from several groups implies that there is a certain degree of plasticity in the current models of haematopoiesis and they are not mutually exclusive. CMPs have been shown to have low B cell commitment potential *in vivo* (Akashi et al., 2000). The progenitor cell microenvironment, such as cytokine availability, and intrinsic factors, such as expression of specific transcription factors, also play a

crucial role in determining cell fate decisions.

1.1.2 Foetal and adult haematopoiesis

The generation of HSCs can be divided into two stages based on the physiological location of blood cell development, cellular morphology and the expression of cell specific genes. Primitive haematopoiesis occurs in the blood islands of the yolk sac (YS) on embryonic day 7 (E7) and later in the aorta-gonad-mesonephros (AGM) from E8-10. The development of nucleated erythroid cells begins between E7 and E8 (Silver and Palis, 1997; Kingsley *et al.*, 2004). These cells express high levels of foetal (ε and ζ) and low levels of adult haemoglobin genes (α and β) and after E8 they become the dominant cells in circulation. Recently, primitive megakaryocytes have also been identified. In comparison with adult megakaryocytes, these cells have reduced ploidy and a higher platelet production (Xu *et al.*, 2001). From E10, primitive haematopoiesis is replaced with definitive haematopoiesis in foetal liver (FL), where it is now the predominant site for embryonic blood production until birth. At E12.5, definitive enucleated erythrocytes begin to develop. In contrast to primitive erythrocytes, these cells only express adult haemoglobin genes.

There are many differences between adult and embryonic haematopoiesis, some of which include expression of cell surface markers (Mikkola and Orkin, 2006), proliferative and differentiation capacity in culture and responsiveness to growth factors (Sanchez *et al.*, 1996; Traver *et al.*, 2001).

1.2 Myelopoiesis

Development of granulocytes and monocytes from multipotential haematopoietic stem cells is referred to as myelopoiesis. Myeloid cells play a pivotal role in initiating an immune and inflammatory response. Monocytes differentiate into macrophages, mainly function to destroy target cells and pathogens by phagocytosis. Dendritic cells can induce T cell mediated immune responses by presenting antigens in the context of major histocompatibility complex (MHC) molecules. Cells of granulocyte origin include neutrophils, basophils and eosinophils all of which have distinctive functions in an inflammatory response. Granulocytes can be identified through the expression of high levels of Gr1 (Ly6G) and Mac1 (CD11b) whereas monocytes express low levels of Gr1 and high levels of Mac1 (Taylor *et al.*, 2003).

1.2.1 Transcriptional control of myelopoiesis

The main transcriptional regulators of myeloid cell development include PU.1 and CCAAT/enhancer binding protein C/EBPα. PU.1 is a member of ETS family of transcription factors that is expressed in B cells, macrophages, mast cells and early erythroblasts. The importance of PU.1 in myelopoiesis was revealed by the generation of *Pu.1* knock out mice, in which most of the ETS DNA binding domain was replaced with the neomycin-resistance gene. These mice were embryonic lethal between days 16.5 and 18.5 of gestation. However, analysis of cells in the foetal livers of embryonic day (E)-14.5 embryos, showed a normal development of erythrocytes and megakaryocytes, but revealed an absence of progenitors for monocytes and granulocytes (CMP and GMP) (Scott *et al.*, 1994). McKrecher *et al*

also generated *Pu.1* knock out mice by inserting the neomycin-resistance gene into the ETS domain (McKercher *et al.*, 1996). These mice lacked mature granulocytes and macrophages in the bone marrow and foetal liver, whereas deletion of *Pu.1* after GMP formation resulted in excess granulocyte production (Dakic *et al.*, 2005). These studies suggest that PU.1 is required for early myelopoiesis and is essential for the regulation of granulocytic terminal differentiation (Olson *et al.*, 1995; Anderson *et al.*, 1998). One mechanism by which PU.1 mediates its function is by inhibiting progenitor cell commitment to other lineages. This process is mainly mediated by downregulating the expression or repressing the activity of lineage specific genes. In this context, PU.1 has been shown to restrict myeloid lineage development by repressing the activity of the megakaryocyte-erythrocyte (Meg-E) specific gene, GATA1. GATA1 mediated activation of its target gene is inhibited by interaction of PU.1 with the GATA1 DNA binding domain, thus preventing binding of GATA1 to DNA and blocking transcription of genes required for commitment to MEP lineage (Zhang *et al.*, 2000).

Another important regulator of myeloid differentiation is C/EBPα, a member of leucine zipper transcription factor family that is expressed in multiple haematopoietic lineages. There are six members of the C/EBP family, all of which affect different stages of haematopoiesis (Antonson *et al.*, 1996; Nerlov *et al.*, 1998). C/EBPε, for example is required for terminal maturation of granulocytes (Yamanaka *et al.*, 1997). Generation of *C/ebpα* deficient mice has revealed the importance and nonredundant role of the protein in myeloid and granulocyte differentiation. Myeloid differentiation was blocked at the myeloblast stage in these mice and the expression of receptors for granulocyte-colony stimulating factor (G-

CSF) and interleukin-6 (IL6) could not be detected on these cells (Zhang *et al.*, 1997). In addition, progenitor cells isolated from these mice failed to form colony-forming unit (CFU)-G colonies *in vitro*. Granulopoiesis was partially restored by retroviral rescue of G-CSF or IL-6 receptor in foetal liver progenitors lacking C/EBPα (Zhang *et al.*, 1998). Monocytes, erythroid cells and lymphocytes were not affected in these mice. Furthermore, forced expression of C/EBPα in the bipotential myeloid cell line, U937, promotes granulocyte differentiation at the expense of monocytic differentiation. C/EBPα has also been shown to induce granulocyte differentiation by affecting granulocyte specific genes such as G-CSF-R, GM-CSF-R, lactoferrin and neutrophil collagenase (Radomska *et al.*, 1998).

1.3 Megakaryopoiesis

The development of megakaryocytes is referred to as megakaryopoiesis. Megakaryocyte-erythroid progenitors (MEPs) are early precursors that have bilineage potential. Depending on the environmental factors and the presence of specific transcription factors, MEPs can differentiate and commit to the megakaryocytic pathway giving rise to early megakaryocyte progenitor cells, megakaryoblasts. During megakaryocyte development, megakaryoblasts undergo endomitosis, a process whereby cells undergo DNA replication without cell division. This is observed in many eukaryotic cells such as plant endosperms, *Drosophila* and mouse trophoblast giant cells (Varmuza *et al.*, 1988; Grafi *et al.*, 1996). However, the biological importance of endomitosis in different cell types is as yet unclear. As the megakaryocyte development progresses the DNA ploidy increases from 8N to 128N, the nuclei become segmented and the cells increase in

size, ultimately resulting in mature megakaryocytes with multi-lobed nuclei. Since the function of megakaryocytes is platelet biogenesis and release into the bloodstream, it is conceivable that endomitosis provides a mechanism for storing high levels of platelets by limiting the use of cellular machinery to platelet production rather than cell division. In this regard, the nuclear polyploidisation can also provide the increase transcriptional capacity required for high levels of platelet production necessary during disease or trauma.

Megakaryocytes represent 0.03-0.06% of all nucleated cells in the bone marrow (Berkow *et al.*, 1984). They are large cells (around 50-70 μm in diameter) and their cytoplasm is composed of a perinuclear zone, containing organelles for protein synthesis, an intermediate zone, which serves as cytoplasmic demarcation membrane thought to be required for platelet production and release, and a peripheral ring of cytoplasm that is free of organelles. Platelet granules are of two types, α-granules and dense bodies. Dense bodies contain nucleotides, calcium and lysosomes whereas α-granules consist of von-Willebrand factor (VWF), factors V, VIII and fibrinogen.

1.3.1 Thrombopoietin (TPO) and its effect on megakaryopoiesis

One of the major regulators of MK development and platelet production is the hormone thrombopoietin (TPO/ c-mpl-ligand/ megakaryocyte growth and development factor (MGDF). The major site of TPO production is in the liver and the kidney. TPO is comprised of two domains based on its homology with the erythroid differentiation factor erythropoietin (EPO). The N-terminus domain has

46% homology with EPO and it is responsible for binding to its receptor c-mpl. In contrast, other than containing N- and O-linked carbohydrate residues, the C-terminus does not show homology with any known protein domains (Bartley *et al.*, 1994; Sohma *et al.*, 1994).

TPO has an important role in the proliferation and survival of megakaryocytes and hence the production of platelets. *Tpo* and *c-mpl* deficient mice have a severe reduction in the level of megakaryocytes and circulating platelets (Gurney *et al.*, 1994). However, the megakaryocytes and platelets that are present are functionally and morphologically normal (Bunting *et al.*, 1997). This suggests that TPO is required for survival and not maturation of megakaryocytes *in vivo*. Multipotential progenitor cells were also reduced in these mice, implying a possible role for TPO in early haematopoiesis (Alexander *et al.*, 1996). Indeed, it was later confirmed that TPO can promote the proliferation and survival of primitive progenitor cells (Ku *et al.*, 1996; Ramsfjell *et al.*, 1997). The effect of TPO on the clonal growth of Lin^- $Sca1^+$ progenitors isolated from BM was investigated by performing semi-solid colongenic assays. These progenitors were shown to have a multilineage potential in the presence of TPO. In addition, TPO, in synergy with the cytokines FLT3 and SCF, increased the growth of multipotent progenitor colonies (Ramsfjell *et al.*, 1996).

Several studies have also highlighted the requirement for TPO in erythropoiesis. TPO in combination with EPO was shown to increase erythroid colony formation of foetal liver and BM derived progenitors (Kobayashi *et al.*, 1995) in addition to progenitors isolated from yolk sac (Era *et al.*, 1997). These studies reflect the

pleiotropic function of TPO in haematopoiesis.

1.3.2 Megakaryocytic cell surface markers

Megakaryocytes can be distinguished from other haematopoietic cells by the expression of the CD41 antigen on the cell surface. CD41 is expressed throughout megakaryocytic development and maturation. The protein is a heterodimer containing a heavy chain glycoprotein IIb-alpha (GPIIb-α) and a light chain glycoprotein IIb-beta (GPIIb-β), linked by a single disulfide bond. CD41 is also expressed on platelets, where the GPIIb-α chain of CD41 interacts with the GPIIIa-β of CD61 to form the platelet glycoprotein complex gpIIb/IIIa (CD41/CD61) (Mitjavila-Garcia *et al.*, 2002). This complex is required for platelet function and aggregation and acts as a receptor for adhesion molecules such as fibrinogen. CD41 is also recognized as a haematopoietic progenitor cell marker. It is expressed on early HPCs derived from both the aorta-gonad-mesonephros (AGM) and the yolk sac and its expression is lost upon differentiation (Mitjavila-Garcia et al., 2002). Since CD41 is expressed on HPCs derived from embryonic stem (ES) cells, it is suggested that its expression indicates an initiation of definitive haematopoiesis during embryoid body (EB) development *in vitro* (Mikkola *et al.*, 2003). This was further supported in the yolk sac *in vivo*, where CD41 expression preceded the expression of the panhaematopoietic marker, CD45. At the foetal liver stage, CD41 expression is downregulated and all definitive haematopoietic cells express CD45 (Mitjavila-Garcia et al., 2002; Mikkola et al., 2003). In adult haematopoiesis, CD41 expression is detected on BFU-E and CFU-MK, and later on megakaryocytes and platelets in the bone marrow.

Another protein expressed on the cells of the megakaryocytic lineage is CD9 which is a member of the tetraspanin family of transmembrane receptors. The expression pattern of CD9 is more complex than CD41. CD9 is expressed on pre-B lymphocytes, basophiles, eosinophils, activated T cells, megakaryocytes and platelets (Cramer *et al.*, 1994; Berger *et al.*, 1996). CD9 upregulation in megakaryocytes occurs prior to the expression of CD41 and CD61 (Le Naour *et al.*, 1997) and its expression is not detected on the cells of the erythroid lineage, making it an important marker to distinguish megakaryocytes from these closely related lineages. In contrast to CD41, CD9 is not expressed on early HSCs. However, an anti-CD9 antibody has been used to isolate and purify megakaryocytic progenitors from the bone marrow stem cell progenitor (c-kit$^+$, Sca1$^-$, IL7R-α^-, Thy1.1$^-$, Lin$^-$) population. This population of cells was shown to be positive for CD41 and gave rise to megakaryocytes and platelets *in vitro* and *in vivo*. (Nakorn *et al.*, 2003)

1.3.3 Transcriptional regulation of megakaryopoiesis

Gene targeting studies in mice have highlighted the importance of various transcription factors required for progenitor cell commitment, proliferation and terminal differentiation of megakaryocytic cells. These transcription factors include members of the GATA1 family (GATA1 and GATA2), BACH1, RUNX1, NF-E2 and ETS family members FLI1 and ETV6. The role of some these vital proteins at the different stages of megakaryocyte development will be discussed below (**Figure 1.2**).

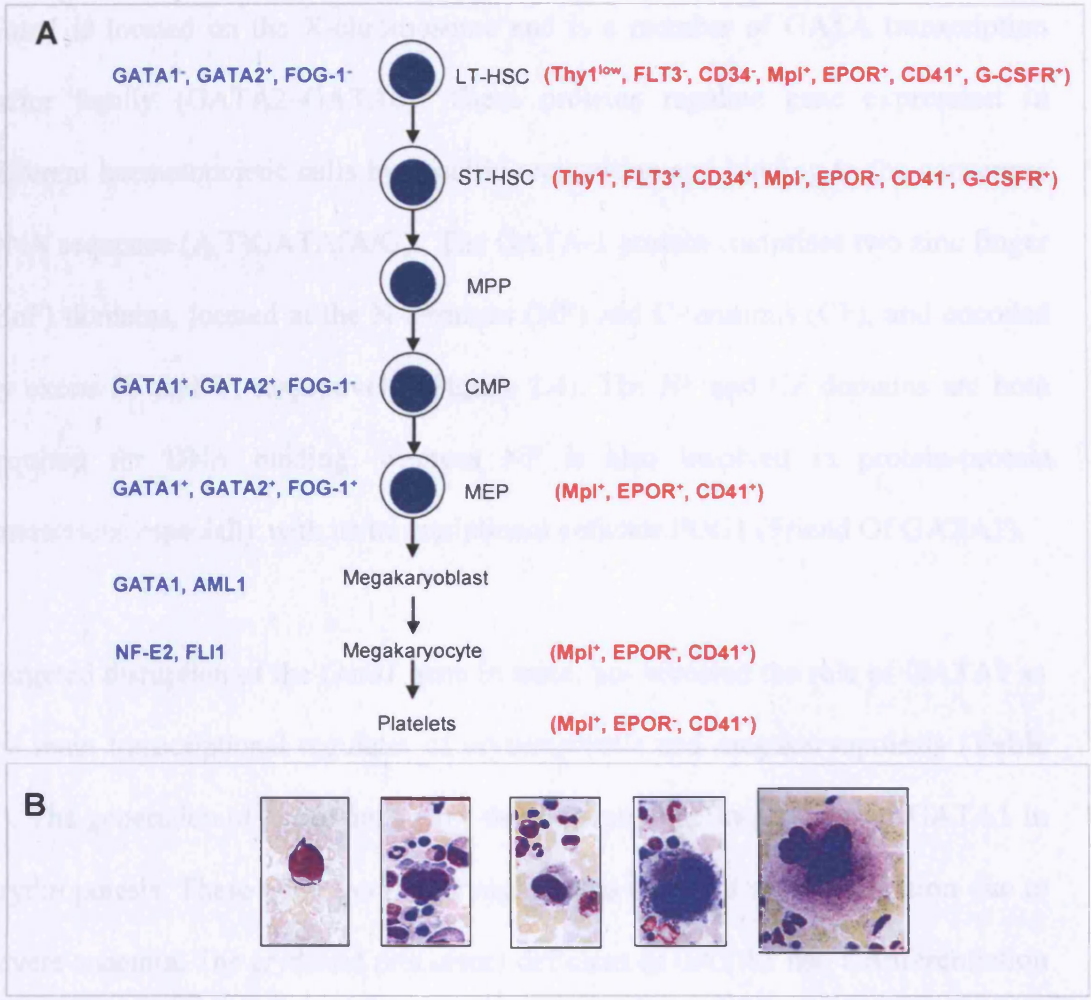


Figure 1.2. Megakaryocyte development. A. Megakaryocyte-erythroid progenitors (MEPs) are bipotential cells capable of commitment to either the megakaryocyte or the erythroid lineage. This lineage commitment is dependent on cytokine conditions and on the level and availability of specific transcription factors. GATA1 and FOG-1 are involved in the commitment of CMPs to the megakaryocyte or erythroid lineage. NF-E2 and FLI1 transcription factors are essential for terminal differentiation, which involves platelet production and release. The cell surface markers that differentiate different population of HSCs, progenitors and mature cells are indicated in red. Thy1, thymus 1; FLT3, FMS-Like Tyrosine Kinase 3; EPOR, Erythropoietin Receptor; CD41, Glycoprotein IIb/IIIa integrin receptor; GCSF-R, G-CSF Receptor. Diagram adapted from Pang *et al* 2005 and Iwasaki *et al* 2003. **B.** The morphology of cells undergoing maturation (Wright-Giemsa stained cells). Megakaryoblasts have a higher nucleus to cytoplasm ratio and as megakaryoblasts differentiate they become larger in size and the nuclei become multi-lobulated (Hoffbrand and Pettit, 1988).

1.3.3.1 GATA1

Gata1 is located on the X-chromosome and is a member of GATA transcription factor family (GATA2–GATA6). These proteins regulate gene expression in different haematopoietic cells by specific recognition and binding to the consensus DNA sequence (A/T)GATA(A/G). The GATA-1 protein comprises two zinc finger (ZnF) domains, located at the N-terminus (NF) and C-terminus (CF), and encoded by exons IV and V, respectively (**Figure 1.4**). The NF and CF domains are both required for DNA binding, whereas NF is also involved in protein-protein interactions especially with its transcriptional cofactor FOG1 (Friend Of GATA1).

Targeted disruption of the *Gata1* gene in mice, has revealed the role of GATA1 as the main transcriptional regulator of erythropoiesis and megakaryopoiesis (**Table 1**). The generation of *Gata1* null mice demonstrated the importance of GATA1 in erythropoiesis. These mice were embryonic lethal and died at mid gestation due to severe anaemia. The erythroid precursors deficient in GATA1 had a differentiation block at the proerythroblast stage of the erythroid development (Fujiwara *et al.*, 1996). As these embryos die between E10.5 and E11.5, the functional importance of GATA1 in the megakaryocyte development could not be investigated in these mice.

The *Gata1* null embryonic stem cells generated by Pevny *et al* also showed the importance of GATA1 in erythroid maturation (Pevny *et al.*, 1991). The resultant chimeric mice were anaemic and the cells failed to contribute to differentiated erythroid cells. Further studies on these mice revealed that lack of GATA1 resulted

in abnormal proliferation of megakaryocytes but that megakaryocyte terminal differentiation, as measured by platelet production, was not affected (Pevny *et al.*, 1995). The authors concluded that the function of GATA1 was compensated for by another family member, GATA2. GATA2 is also expressed in megakaryocyte cells, but expressed at very low levels in early erythropoiesis, explaining the non-redundancy in the erythroid cells.

Lineage specific deletion of GATA1 has demonstrated its importance in megakaryocyte development. In the *Gata1*$^{neo\Delta HS}$ mice, sequences including the DNaseI hypersensitivity (HS) sites present upstream of the GATA1 promoter were replaced with a neomycin-resistance cassette by homologous recombination in ES cells (McDevitt *et al.*, 1997). The level of GATA1 expression in erythroid cells was reduced by 5 fold, but its expression was not detected in megakaryocytes. These mice exhibit primitive erythrodysplasia and definitive erythroid cells are arrested at the proerythroblast stage. Most mice died during embryogenesis due to anaemia and thrombocytopenia.

In a second *Gata1*$^{\Delta neo\Delta HS}$ gene targeted mutant mouse, the neomycin cassette was removed by CRE-mediated site specific recombination, which restored GATA1 expression in erythrocytes (Shivdasani *et al.*, 1997). The reduced levels of GATA1 in erythrocytes of *Gata1*$^{neo\Delta HS}$ mice were attributed to the transcriptional interference caused by the insertion of neomycin cassette in a transcriptionally active region. Since GATA1 expression was normal in erythroid cells, the *Gata1*$^{\Delta neo\Delta HS}$ mice were not anaemic and did not exhibit any abnormalities in the erythroid lineage. In common with the *Gata1*$^{neo\Delta HS}$ mice, GATA1 expression was

undetectable in megakaryocytes of these mice. There was a profound reduction in the number of circulating platelets and thrombocytopenia was manifested in these mice as a consequence. In contrast to erythroid cells, there was an increase in the levels of immature megakaryocytes in the spleen and bone marrow and colony forming assays using progenitor cells isolated from the yolk sac and foetal liver resulted in hyperproliferative colonies which represent the accumulation of megakaryoblasts (Shivdasani et al., 1997). There were however, some mature megakaryocytes present in these mice, although these were shown to be abnormal in size and in their ability to undergo endomitosis. Furthermore, the platelets produced by these megakaryocytes were functionally defective as shown by the prolonged bleeding in these mice upon exposure to trauma (Vyas *et al.*, 1999). These studies suggest that GATA1 is not essential for the initial stages of megakaryopoiesis.

The role of GATA1 in terminal megakaryocyte maturation and primitive erythropoiesis was further confirmed by the analysis of the *Gata1.05* mouse line. The erythroid specific regulatory elements of the *Gata1* gene, were disrupted by the insertion of the neomycin cassette. Since *Gata1* is an X-linked gene, males only have one copy of the gene and are therefore hemizygous for the *Gata1* gene. The male embryos express GATA1 at 5% of the total mRNA level expressed in wild-type embryos and hence the mouse line is referred to as *Gata1.05*. The resultant mutant mice had a 20 fold reduction of GATA1 expression in erythroid cells. In common with the $Gata1^{neo\Delta HS}$ mutant line and the *Gata1* null embryonic stem cell model, erythroid development was arrested at the proerythroblast stage in *Gata1.05* mice. As a result, hemizygous males are embryonic lethal at E12.5 due to severe

anaemia. Some of the heterozygous females survived but died of a myelodysplastic syndrome (MDS) by 5 months of age (Takahashi *et al.*, 1997; Takahashi *et al.*, 1998). Although megakaryopoiesis appeared normal and the cells stained normally with acetylcholinesterase, terminal differentiation appeared defective due to the reduction in platelet levels, which was reflected in thrombocytopenia in these mice.

Table 1: Summary of GATA1 gene-targeted mice

GATA1 mice	Targeting strategy	MK	Ery	Phenotype
$GATA1^{null}$	Total GATA1 KO	×	×	- Embryonic lethal (E10.5)
$GATA1^{neo\Delta HS}$	**Replacement of sequences upstream of GATA1 promoter with a neomycin cassette**	×	↓ 5F	**- Most die during embryogenesis** **- 5% of the hemizygous males are born anaemic and thrombocytopenic** **Few animals that survive recover from anaemia but remain thrombocytopenic**
$GATA1^{\Delta neo\Delta HS}$	Deletion of the neomycin cassette from the $GATA1^{neo\Delta HS}$	×	✓	- Thrombocytopenia - Increased levels of immature MK - Abnormal differentiated MK
GATA1.05	**Disruption of erythroid specific sequences in the promoter of GATA1**	×	↓ 20F	**- Hemizygous males die by E12.5** **- Heterozygous females exhibit anemia and thrombocytopenia and begin to die by 5 months**
GATA Val205Gly	Mutation in FOG1 interaction domain	✓	✓	- Hemizygous males die by E12.5

MK: megakaryocyte, Ery: Erythrocyte, KO: Knock out, F: Fold

Adapted from (Gurbuxani *et al.*, 2004)

1.3.3.2 FOG1

The function of GATA1 in erythroid and megakaryocyte development is partly dependent on the interaction with its cofactor FOG1 (Friend Of GATA1). FOG1 is a 9 zinc finger protein that can interact with the N-terminus zinc finger of all GATA family members via its 6th zinc finger (Fox *et al.*, 1998). *Fog*$^{-/-}$ mice had a marked defect in erythroid development with maturation arrest at the proerythroblast stage and died at E10.5-E12.5. This resembles the phenotype detected in the *Gata1*$^{-/-}$ mice, suggesting that both GATA1 and FOG1 proteins are required for effective erythropoiesis. Several groups have generated *in vivo* models to demonstrate the importance of the GATA-FOG interaction in megakaryopoiesis.

In contrast to the *Gata*$^{\Delta neo\Delta HS}$ mice where only megakaryocyte terminal maturation was affected, the *Fog*$^{-/-}$ mice failed to produce any megakaryocytes, suggesting a GATA1 independent function of FOG1 in early megakaryopoiesis (Tsang *et al.*, 1997). Alternatively, as GATA2 is expressed in primitive HSCs and in megakaryocytes, it is possible that in *Gata1*$^{\Delta neo\Delta HS}$ mice GATA2 interacts with FOG1 and compensates for the function of GATA1 in early megakaryopoiesis. This hypothesis was confirmed by the generation of a double knock-in mice containing a mutation (Valine-to-Glycine substitution) in the GATA1 and GATA2 motif required for interaction with FOG1 (Chang *et al.*, 2002). This mutation is identical to the natural occurring missense mutation in the GATA1 N-terminus zinc finger domain identified in patients with X-linked thrombocytopenia and anaemia (Nichols *et al.*, 2000). Yolk sac derived progenitors from these double knock-in mice (*Gata*^{V205G}) failed to form any megakaryocytic colonies thus suggesting that

the function of FOG1 in early megakaryopoiesis is GATA dependent (GATA1 or GATA2).

It has also been demonstrated that the GATA1-FOG1 interaction is crucial for the late stages of megakaryopoiesis (Chang et al., 2002; Shimizu *et al.*, 2004; Muntean and Crispino, 2005). Shimizu *et al* showed that although the *Gata1*^{V205G} cDNA transgene rescued the *Gata1.05* mutant mice from embryonic lethality, the dysregulated megakaryocyte terminal maturation in adult mice could not be rescued. In this regard, expression levels of genes required for late stages of megakaryocyte differentiation, such as NF-E2 and MAFK, were severely reduced (Shimizu et al., 2004). This study suggests that the GATA-FOG interaction is dispensable for early erythropoiesis but it is vital for regulating genes required for megakaryocyte differentiation and platelet formation. This study also highlighted the fact that some GATA1 mediated gene regulation may be FOG1 independent, as shown by the altered expression of some genes in the rescued mice.

1.3.3.3 GATA2

In common with GATA1, GATA2 is expressed in mast cells, erythroid cells and megakaryocytes. In contrast to GATA1, GATA2 is expressed in early HSCs and current data suggests that this protein is critical for survival and proliferation of primitive HSCs (Ling *et al.*, 2004). In addition, expression of GATA2 protein at different developmental stages, is essential for lineage commitment and differentiation. The generation of *Gata2* deficient mice revealed the importance of GATA2 in erythropoiesis, as these have low numbers of erythroid cells and died at E10-E11 gestation (Tsai *et al.*, 1994). Indeed, GATA2 expression decreases during

erythroid terminal differentiation and enforced expression of the protein in primary erythroblasts results in growth and differentiation arrest at the proerythroblast stage. Furthermore, over-expression of GATA2 in the K562 erythroleukemic cell line was shown to inhibit erythroid differentiation and promote megakaryocyte differentiation (Ikonomi *et al.*, 2000). GATA2 alone is not required for megakaryocytic development since in vitro differentiated *Gata-2*$^{-/-}$ murine ES cells produce normal megakaryocytes (Tsai and Orkin, 1997). However, GATA2 can compensate for the function of GATA1 in early but not late megakaryopoiesis (Chang et al., 2002).

1.3.3.4 NF-E2 and FLI1 are required for terminal maturation of megakaryocytes

Megakaryocyte terminal differentiation is characterised by the change in cellular morphology and proplatelet formation and release, all of which are accompanied by an increase in expression levels of genes required for these processes. Nuclear factor-erythroid 2 (NF-E2) is a heterodimeric protein that regulates the terminal maturation of megakaryocytes and platelet biogenesis. Expression of erythroid specific globin genes are mediated by core enhancers, such as NF-E2 motifs within the locus control region (LCR). The NF-E2 heterodimeric complex is the major protein that binds these sites. The NF-E2 complex is an heterodimer of two basic leucine zipper (bZIP) proteins, a large p45 NF-E2 subunit belonging to a family closely related to the *Drosophila* Cap-N-Collar (CNC) family, and the smaller subunit, p18 NF-E2 or MAFK, belonging to the small MAF subfamily. The expression of p45 NF-E2 is restricted to the haematopoietic tissues whereas p18 NF-E2 is widely expressed. The p45 subunit can bind to NF-E2 sequences by

forming heterodimers with MAFK protein and the other small MAF proteins, MAFF and MAFG, however, the NF-E2 complex containing MAFK and p45 display the highest affinity to NF-E2 binding sites (Igarashi *et al.*, 1994; Meguro *et al.*, 1995). The role of NF-E2 in late stages of megakaryooiesis was confirmed by the phenotype of mice deficient in the p45 subunit of NF-E2. These mice lack terminally differentiated megakaryocytes and circulating platelets and as a result die of haemorrhage (Shivdasani *et al.*, 1995; Lecine *et al.*, 1998). The MAFK subunit of NF-E2 also plays a role in the regulation of megakaryocytic differentiation, although only in synergy with another family member, MAFG. This is based on reports that mice lacking *MafK* did not exhibit any abnormalities in the megakaryocyte lineage and were otherwise viable and healthy. In contrast, mice deficient in *MafG*, where the entire coding sequence was replaced with *Escherichia coli lacZ* gene, had abnormal megakaryopoiesis as shown by impaired platelet formation and increased megakaryocyte proliferation (Shavit *et al.*, 1998). However, the phenotype of the *MafG* mutant mice was exacerbated when combined with the *MafK* heterozygotes ($MafG^{-/-}/MafK^{+/-}$). These mice exhibited profound thrombocytopenia and megakaryocytes were defective in proplatelet formation (Onodera *et al.*, 2000). These results suggest a dosage dependent function for MAFK, in platelet biogenesis.

Fli1 (Friend leukemia virus integration 1), a member of the ETS family of transcription factors, is also required for effective megakaryocytic differentiation. Mice lacking *Fli1* display dysmegakaryopoiesis. These mice die at mid gestation and *in vitro* analysis of megakaryocyte development has revealed abnormalities in cellular morphology and a block in megakaryocytic maturation (Hart *et al.*, 2000).

The expression of the late megakaryocyte specific gene, glycoprotein IX (*GPIX*), was markedly reduced in *Fli*$^{-/-}$ megakaryocytes, whereas the expression of early genes, *cMpl* and *CD41*, remained unchanged. One mechanism of *Fli1* gene regulation may be through its interaction with GATA1 zinc fingers, as this interaction was found to mediate the activation of genes associated with megakaryocyte maturation, such as *GPIX* (Eisbacher *et al.*, 2003). Observations from Paris-Trousseau syndrome (PTS) patients, further imply a role for FLI1 in megakaryopoiesis. Terminal deletion of the long arm of chromosome 11, results in the deletion of the *Fli1* gene. As a consequence, these patients suffer from severe thrombocytopenia and there is an abnormal accumulation of immature megakaryocytes (Raslova *et al.*, 2004).

1.4 Acute childhood leukaemia

Human acute leukaemias can be characterised by the abnormal proliferation or differentiation of cells of the haematopoietic system with the subsequent clonal accumulation of immature cells or blasts in the bone marrow (BM) and peripheral blood (PB). Acute leukaemias can be subdivided into either acute lymphocytic leukaemia (ALL) or acute myelogenous leukaemia (AML), which affect cells of the lymphoid or the myeloid lineage, respectively. ALL is the most common form of childhood malignancy accounting for approximately 70% of childhood leukaemias, whereas around 30% of children with leukaemia have AML. Chronic myelogenous leukaemia (CML) is also observed in children but with a much lower incidence. Myeloid leukaemias can be divided into subgroups based on the type of myeloid cells affected and the chromosomal aberrations associated with the disease (**Table**

2).

Table 2: French-American-British (FAB) classification of AML

FAB Type	Description	Mutations
M0	Undifferentiated	AML1, Myeloperoxidase negative; myeloid markers positive
M1	Myeloblastic without maturation	Some evidence of granulocytic differentiation
M2	Myeloblastic with maturation	t(8;21) AML1-ETO and AML1-EVI1
M3	Promyelocytic	t(15;17) PML-RARα or other - RARα translocations
M4	Myelomonocytic	11q23 translocations t(9;11) MLL-AF9 and t(2;11) HOXD13-NUP98
$M4_{E0}$	Myelomonocytic with bone marrow eosinophilia	Inversion of chromosome 16 involving CBFβ
M5	Monocytic	11q23 translocations t(9;11) MLL-AF9 and t(8;16) MOZ-CBP
M6	Erythroleukemia	
M7	Megakaryoblastic	GATA1 mutations in those associated with DS, t(1;22) RBM15-MLK associated with non DS-AMKL

Table from (Tenen, 2003).

1.4.1 Models of leukaemic initiation and progression

There are currently two proposed models of leukaemic initiation and progression. The stochastic model predicts that all the cells in the leukaemic clone have the potential to give rise to leukaemia and are therefore homogenous. According to this model, genetic changes that cause leukaemia can be detectable in every cell population. The stochastic model of leukaemic initiation is the basis of most of the current therapeutic approaches, which are designed to target all the cells in the

leukaemia, especially proliferating blasts. In contrast, the stem cell model postulates that there is a heterogeneous population of cells, each having different self renewal and differentiation capacities. According to this model, leukaemic cells have their own stem cells, capable of initiating and maintaining the growth of the leukaemia. These leukaemic stem cells (LSC) retain many properties of stem cells including self renewal, proliferative and differentiation potential. This model proposes that LSCs can be purified from the total leukaemic cell population based on distinctive expression of cell surface marker.

The first experimental evidence in support of the stem cell model was provided by AML xeno-transplantation into immune deficient recipient SCID and NOD/SCID mice. It became clear that a subset of cells termed SCID leukaemia initiating cells (SL-ICs), were capable of maintaining the leukaemic clone in recipient mice (Lapidot *et al.*, 1992). These cells demonstrated high self renewal capacity in the immune deficient NOD/SCID mice. In these xeno-transplantations studies, only $CD34^{+}CD38^{-}$ HSCs from all AML subtypes, except for AML-M3, were capable of transplanting leukaemia in NOD/SCID mice (Bonnet and Dick, 1997). This suggests the existence of a hierarchy in AML, as in normal haematopoiesis. Further characterisation of these cells revealed differences in the expression of cell surface markers between normal HSCs and LSCs. Expression of the IL-3 receptor α chain (IL-3Rα), for example, is confined to LSCs, whereas c-Kit and Thy-1 are expressed on HSCs and are lacking on LSCs (Blair and Thomas, 1997; Blair and Athanasiou, 2000; Jordan, 2002). Further understanding of the distinctive biological features of LSCs and normal HSCs would allow for the development of therapeutic approaches to specifically target the leukaemic initiating cells in paediatric leukaemia.

Many studies have addressed the nature of the target cell in the AML transforming events. The most obvious candidate cells are the uncommitted HSCs since these cells have a high self renewal capacity which removes the necessity to activate a self renewal program by the transforming mutations. Furthermore, the longevity of HSCs could allow for the acquisition of additional genetic events required for leukaemic transformation. The possibility that HSCs are target cells in AML was supported in mouse transplantation assays. Transduction of HSCs by the *MLL-GAS7* fusion gene induced mixed lineage leukaemia in transplanted mice, but not with CMP or GMP transduced cells (So *et al.*, 2003). A study by Miyamoto *et al* demonstrated that although HSCs may be a target cell for a specific transforming event, it is the subsequent changes in a more committed progenitor that gives rise to LSC (Miyamoto *et al.*, 2000).

The hypothesis that the primary leukaemic event occurs in the HSCs, with high self renewal capacity, was challenged by the discovery that leukaemic transformation can also occur in a more committed progenitor cell, where the self renewal potential is reactivated by the transforming mutation. Thus, the *MLL-ENL* fusion gene was shown to give rise to the same leukaemia independent of whether the target of transformation was HSC, CMP or GMP (Cozzio *et al.*, 2003). In addition, the *MOZ-TIF2* fusion gene associated with AML was shown to transform both HSCs and CMPs and GMPs (Huntly *et al.*, 2004). These studies suggest that depending on the type of transforming mutation, LSC can be generated from either progenitor cells or a more primitive HSC.

1.5 Down syndrome

The hallmark of Down syndrome (DS) is the acquisition of an extra copy of human chromosome 21 (Hsa21/ *Homo sapien* 21)/ trisomy 21. Trisomy 21 occurs in 1 out of 700 live births and is the only autosomal aneuploidy that is not embryonic lethal and is associated with postnatal survival. The pathology of DS affects multiple systems in an individual ranging from the nervous system to immune system abnormalities and an aberrant haematopoietic system. The occurrence and severity of the clinical features of DS are highly variable amongst individuals. Phenotypic characteristic of DS include, broad hands, hypotonia, short stature, and craniofacial dysmorphology such as flat nasal bridge and open mouth. There are two congenital diseases that affect DS individuals. Congenital heart defects (CHD) occur in ~50% of all DS newborns, whereas, the congenital gastrointestinal tract (CGI) dysfunction is detected in 4-5% of newborns. Both disorders can be fatal if left untreated. DS individuals suffer variable pathological penetrance in the nervous system. Patients with DS have delayed cognitive development and mild to moderate mental retardation. Furthermore, individuals over the age of 30 commonly exhibit pathological abnormalities correlated with Alzheimer's disease (AD). Other clinical manifestations of DS disorder include infertility, which mostly affects men, and thyroid dysfunction.

Detection of solid tumours is extremely rare in DS. There are a few reported cases of DS patients with retinoblastoma (RB), however, evidence based on population studies indicate that there is no significant correlation between DS and RB (Hasle *et al.*, 2000; Patja *et al.*, 2006). Incidence of other solid tumours such as Wilms

tumour and neuroblastoma, is lower in DS patients compared with the general population (Satge *et al.*, 1998). Less frequently, Ewing's sarcoma has been associated with DS. On the other hand, the haematopoietic system is severely affected in DS individuals. There is a high incidence of childhood leukaemias, such as acute myeloid leukaemia (AML), and in particular, acute megakaryoblastic leukaemia (AMKL).

1.5.1 The origin of trisomy 21: Chromosome recombination and non-disjunction

Aneuploidy (trisomy or monosomy) is the most common chromosomal abnormality identified in humans. Events occurring during the meiotic division are a contributing factor to the development of aneuploidies. Meiosis involves two nuclear divisions after a single phase of DNA replication. During the first meiotic division (MI), which is dominated by prophase I, the two sets of homologous chromosomes each consist of two sister chromatids. Chromosomal recombination occurs between non sister chromatids at chiasmata (point at which the exchange of genetic material occurs on the chromatids). Following the chromosomal crossover events, homologous chromosomes are segregated into two separate cells thereby reducing the number of chromosomes from $4n$ to $2n$. The second meiotic division (MII) involves the alignment of chromosomes on a second spindle without further DNA replication and separation of sister chromatids into four haploid cells. Failure of homologous chromosomes to separate during meiosis is referred to as chromosome non-disjunction. This results in haploid cells with either an additional or reduced copy of a particular chromosome.

Chromosomal non-disjunction in lower organisms, such as *Saccharomyces cerevisiae* and *Drosophila*, is well documented. Isolation of mutations in *Drosophila* has revealed a reduction in recombination, in particular, in the distal regions and premature segregation of homologous chromosomes at MI (Carpenter and Sandler, 1974). Mutations in genes such as the *Ord* and *Mei*-S332 in *Drosophila* result in the premature disjoining of sister chromatids in MI at prometaphase I and anaphase I, respectively (Miyazaki and Orr-Weaver, 1992).

The mechanism(s) underlying chromosome non-disjunction in humans has been under intense investigation. The hypothesis that alterations in recombination are a critical factor in human trisomy was initially proposed by Henderseon and Edwards in 1968 (Henderseon and Edwards 1968). A study carried out by Warren *et al* measuring the recombination frequency on chromosome 21, provided the first evidence correlating reduced recombination with human trisomy 21. This reduction was not due to failure or absence of any homologous chromosome 21 exchange at meiosis I (MI), as initially predicted (Warren *et al.*, 1987; Sherman *et al.*, 1991). The genetic linkage map of chromosome 21 revealed that the number of chiasmata between non-sister chromatids may play a crucial role in efficiency of recombination (Sherman *et al.*, 1994). It later became apparent that efficient separation of chromosomes appears to be influenced by the position at which the exchange occurs. There is a reduced efficiency of chromosome segregation when exchange occurs at distal 21q, or telomeric region, of the chromosome at MI, in comparison with centromeric exchanges. Errors at MII are also linked with chromosomal non-disjunction. In contrast to MI, there is an increased recombination in proximal 21q, pericentromeric region, of the chromosome at MII

(Lamb *et al.*, 1996). It is as yet unclear if MII non-disjunction originates in MI or if it is an event specific to MII.

Sherman and colleagues provided the first evidence for a link between maternal age and reduced recombination events at MI. Studies of 300 DS individuals of varied ages revealed a reduction in recombination along 21q with increased maternal age (Sherman et al., 1994). Chromosome 15 non-disjunction and recombination events have also been shown to be associated with maternal age (Robinson *et al.*, 1998). A two hit hypothesis has been proposed to explain the link between chromosomal non-disjunction and maternal age. The first hit involves age-independent events that occur in MI with the establishment of a chromosome configuration that is susceptible to non-disjunction. The second hit requires degradation of meiotic cell cycle machinery which prevents effective processing or segregation of non-sister chromatids. The defective meiotic process is associated with maternal age and as yet unknown environmental factors.

It is now believed that reduced recombination at MI is a common source of human aneuploidy. The origin and possible cause of chromosome nondisjunction varies in trisomy 16, 18, 15, 22 and paternally and maternally derived sex chromosomes. For example in maternally derived trisomy 22, most cases involve MI nondisjuntion, whereas for trisomy 18 most cases are linked with errors at MII.

1.5.2 Murine models of Down syndrome

Genetic mapping of DS individuals who shared clinical features led to the identification of a common region on chromosome 21, referred to as the Down syndrome critical region (DSCR). It is proposed that genes within this region contribute to the pathogenesis of various DS phenotypes. The initial reports were based on the analysis of patients with partial trisomy 21, where patient's clinical phenotypes were assigned to specific segment in chromosome 21 (Korgaonkar *et al.*, 2005). This region extends about 5 Mb from *D21S17* to *Mx1* and clinical features linked to this region include mental retardation, craniofacial anomalies and short stature. However, there is currently a discrepancy in the location of the DSCR within chromosome 21, since several reports have associated DS clinical manifestations to different regions on HSA21. This has made it difficult to correlate a certain phenotype associated with DS to a single "critical chromosomal region". Several murine models of DS have been generated in order to attempt to link these genes on chromosome 21 to different clinical traits of DS. The orthologues of genes located on HSA21 are located on three separate mouse chromosomes (Mmu) 16, Mmu17 and Mmu10. The conserved genes are distributed on different regions of the chromosomes (**Figure 1.3**).

Two transgenic murine models, Ts65Dn and Ts1Cje, partially trisomic for MMu16, have been generated. The Ts65Dn segment is comprised of genes from the *App* (amyloid beta (A4) precursor protein) gene up to the *Znf295* (zinc finger protein 295) gene (Davisson *et al.*, 1990). Phenotypes in the Ts65Dn mice that recapitulate those found in human DS, include abnormal development of the cerebellum (Baxter

et al., 2000) and reduced development of craniofacial skeleton. These mice also develop myeloproliferative disorders characterised by thrombocytosis, bone marrow fibrosis and anaemia (Kirsammer *et al.*, 2008). In the TsCje mice, the extra copy of chromosome 16 spans from the *Sod1* (21q22.1) to the *Mx1* (21q22.3) region. However, the *Sod1* gene is functionally inactive due to the insertion of a neomycin resistance gene (**Figure 1.3**). In comparison with Ts65D, the deficiency in learning is much less pronounced in the Ts1Cje model, and degeneration of the basal forebrain cholinergic neurons is absent (Sago *et al.*, 1998).

O'Doherty *et al* have generated the trans-chromosomic mouse line TC1 that closely reflects the genetic situation in human DS (O'Doherty *et al.*, 2005). These mice carry a copy of HSA21 and are therefore trisomic only for the genes expressed on this chromosome. The other murine models generated to date comprise of a limited percentage of the orthologs of genes present on HSA21. In contrast, TC1 mice contain 92% of the genes on HSA21, making it the most applicable model system to study the pathology of DS. The human-mouse tranchromosomic embryonic stem (ES) cell line, lacked two segments of HSA21 (O'Doherty et al., 2005). One region spans 3.4 Mb from *CXADR* and *D21S1922* and the second region spans 1.5 Mb from *IFNAR1* and *RUNX1*. Abnormalities detected in mice generated from these ES cells, include learning difficulties and a reduction in cerebella neuron density. The most striking feature of these mice is the occurrence of congenital heart defects which are a prominent clinical manifestation in human DS. This phenotype is rarely observed in other mouse models. However, in contrast to Ts65Dn and Ts1Cje, the TC1 mice did not display any defects in the craniofacial morphology (O'Doherty et al., 2005).

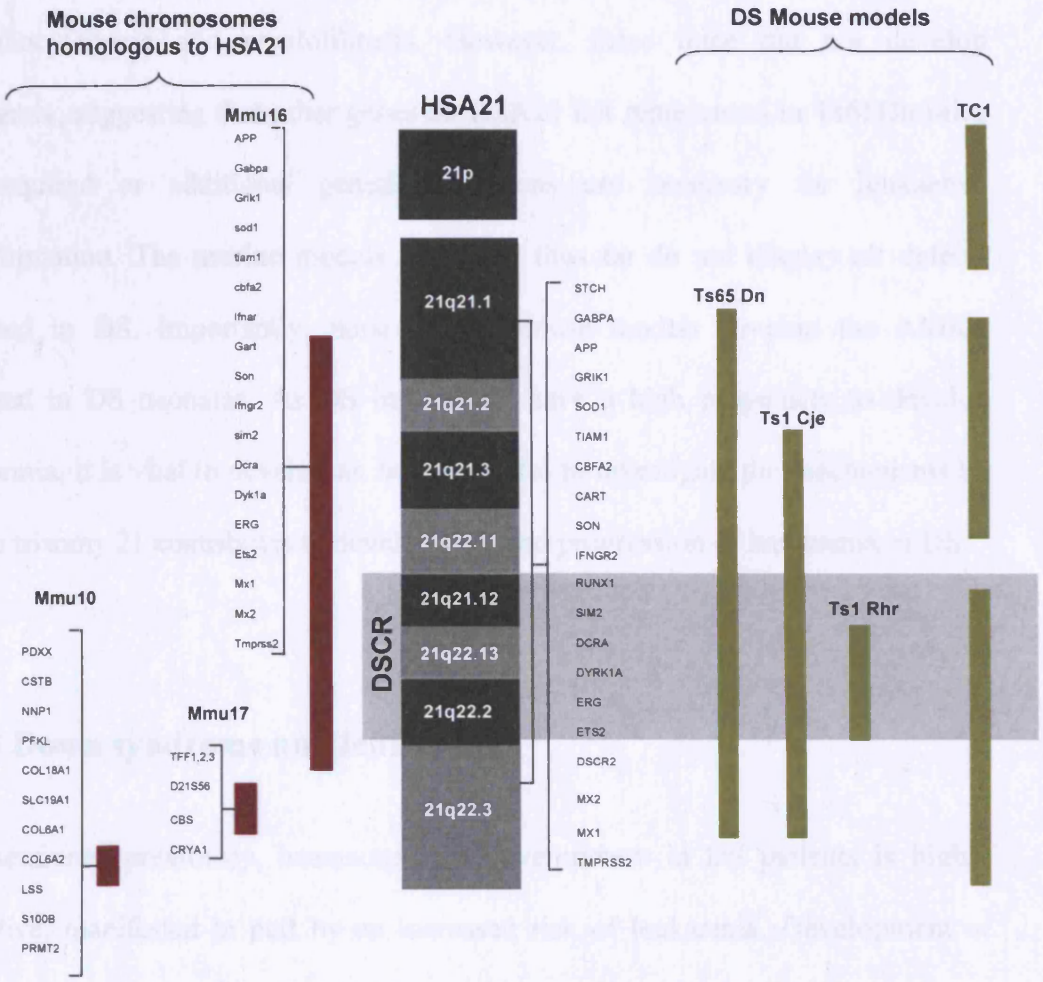


Figure 1.3. Murine models of Down syndrome (DS). The genes located on the human chromosome 21 are located on three separate mouse chromosomes, Mmu10, Mmu16 and Mmu17. The red bars represent the region of the mouse chromosome and the genes located on the chromosomes that are orthologous to the human chromosome 21 genes. The genes located within the Down syndrome critical region (DSCR) are highlighted in grey. Several DS murine models have been generated. The green bars represent the genes that are trisomic in these mouse models. Ts65Dn, Ts1 Cje and Ts1 Rhr mouse models are trisomic for mouse chromosome 16. The TC1 model uses the human chromosome 21. There are two gaps within HSA21 in the TC1 mice which results in absence of 8% of the genes expressed on this chromosome. Diagram adapted from Reeves *et al.*, 2001 and Arron *et al.*, 2006).

Recent analysis of the haematopoietic system in Ts65Dn mice showed that these mice developed a myeloproliferative disease (MPD) (Kirsammer et al., 2008). The characteristic features of MPD include megakaryocytic dysplasia, thrombocytopenia and myelofibrosis. However, these mice did not develop leukaemia, suggesting that other genes on HSA21 not represented in Ts65Dn mice are required or additional genetic mutations are necessary for leukaemic transformation. The murine models generated thus far do not display all defects detected in DS. Importantly, none of the mouse models develop the AMKL detected in DS neonates. As DS individuals have a high propensity to develop leukaemia, it is vital to develop an *in vivo* model to investigate the mechanisms by which trisomy 21 contributes to development and progression of leukaemia in DS.

1.5.3 Down syndrome and leukaemia

As mentioned previously, haematopoietic development in DS patients is highly defective, manifested in part by an increased risk of leukaemia. Development of leukaemia is more prevalent in children and the risk decreases with age (Hasle et al., 2000). There is a 20 fold increased chance of leukaemia in DS compared with non-DS patients, and in particular there is a 600-fold increase risk for acute megakaryoblastic leukaemia (AMKL) development in these children. Leukaemia usually occurs within the first 3-4 years of life and around 10% of children with DS are born with a disorder referred to as transient myeloproliferative disorder (TMD) / transient abnormal myelopoiesis (TAM) / transient leukaemia (TL).

TMD is characterised by the abnormal accumulation of myeloblasts in the

peripheral blood (PB) and bone marrow (BM). TMD can be distinguished from AML by clinical presentation, characteristic cytogenetic and haematological abnormalities and by its spontaneous resolution, typically in the first 3 months of life. TMD is usually diagnosed during the first few days of life and AML generally occurs after 1 year of age (Hayashi *et al.*, 1988). The blasts associated with TMD often exhibit AMKL morphology, expressing megakaryocytic and sometimes erythroid markers. In most cases, trisomy 21 is the sole chromosomal abnormality (Massey *et al.*, 2006), whereas blasts in AML may show complex cytogenetic abnormalities. AML blasts express high levels of myeloid markers in comparison with the TMD blasts (Karandikar *et al.*, 2001). In the majority of cases, TMD resolves spontaneously in the first three months of life. However approximately 20% of patients with TMD will subsequently progress to develop FAB M7 AMKL. It is generally believed that the occurrence of TMD may be under diagnosed. This has been mainly attributed to the fact that blood counts are not performed on every DS neonates and therefore many cases are left unreported.

AMKL is characterised by the proliferation and accumulation of megakaryoblasts in the BM. The differentiation block at the early stages of the megakaryocytic development causes reduced production of platelets which is manifested as thrombocytopenia in the affected patients. AMKL usually presents with one of two genotypic abnormalities, either Down Syndrome accompanied by the somatic *GATA1* mutation or non-DS AMKL associated with the translocation t(1;22) involving *RBM15* (RNA Binding Motif protein 15) and *MKL* genes. AMKL patients with DS have a much better prognosis than *de novo* AMKL or patients with secondary AMKL.

Children with DS also have an increased risk of developing ALL and AML. There are some differences between the pathogenesis and clinical features of ALL-DS and ALL-non-DS patients. There is a marked difference in the age of onset. Whereas, ALL commonly occurs in neonates, ALL in DS is not manifested in patients less than 1 year of age. There is also a reduced occurrence of chromosomal translocations, such as the t(9;22) and t(4;11), in the presence of trisomy 21 (Dordelmann *et al.*, 1998; Whitlock *et al.*, 2005).

Understanding the mechanism by which DS neonates develop AMKL is under intense investigation. Mutations in the GATA1 gene and genes located in the DSCR have been associated with leukaemogenesis in DS.

1.6 GATA1 mutations

1.6.1 GATA1 mutations in haematopoietic disorders

Studies of families suffering from dyserythropoietic anaemia with thrombocytopenia, X-linked thrombocytopenia and X-linked thalassemia with thrombocytopenia, have led to the identification of mutations in the exon 4 of the *GATA1* gene which encodes the N-finger domain of the GATA1 protein. As this region is involved in protein-protein interactions and GATA1 DNA binding stabilisation, acquisition of mutations could interfere with the normal function of GATA1. Indeed, these inherited mutations have been shown to affect different amino acid residues, which ultimately interfere with the interaction of the GATA1 protein with FOG1, albeit at varying levels. These studies highlight the importance of the GATA1-FOG interaction in the regulation of megakaryocytic and erythroid

cell development.

The V205M mutation, which causes the substitution of methionine for valine at amino acid 205 of GATA-1, was isolated in a patient with dyserythropoietic anaemia (Nichols et al., 2000). The off-spring of this patient also suffered from abnormalities of erythroid as well as megakaryocytic cells. The V205M GATA1 was ectopically introduced in GATA1 null erythroid line G1E. Immunoprecipitation experiment showed that the V205M mutation severely reduced the interaction of GATA1 with its cofactor FOG1 (Nichols et al., 2000). In addition, the expression of mutant GATA1 in cell lines that are GATA1 deficient failed to rescue the erythroid maturation defect, whereas introduction of wild-type GATA1 lead to erythroid differentiation. The D218G mutation was found in the context of X-linked thrombocytopenia. Patients had defective megakaryocytic development, as exhibited by abnormal platelet size, reduction in expression of the of GATA1 target genes, *GP1bα* and *GP1bβ* in the platelets of patients (Freson *et al.*, 2002). This suggests an important role for the GATA1-FOG1 interaction in megakaryocytic maturation. Other mutations, identified in the N-finger of GATA1, include D218Y and G208S, which strongly and weakly reduce the affinity of GATA1 for FOG-1, respectively (Mehaffey *et al.*, 2001; Freson et al., 2002). The R216Q mutation, associated with macro thrombocytopenia and β-Thalassemia, is the only mutation identified thus far that reduces the sequence specific DNA binding of GATA1 (Yu *et al.*, 2002). This mutation also results in a weaker interaction with FOG-1 in comparison with the wild-type GATA1.

1.6.2 GATA1s, the DS-associated GATA mutation

Mutations located at the N-terminus of the GATA1 protein that do not impair FOG1 binding or DNA binding activity but lead to the truncation of the GATA1 transactivation domain, occurs in all DS-AMKL patients. These somatic mutations (missense, deletions, duplications or insertions) introduce a premature stop codon at methionine 1 in exon 2 or interfere with the alternative splicing of exon 2. This leads to initiation of translation from exon 3, replacing full length GATA1 with a shorter isoform, GATA1s (**Figure 1.4A**). The net effect of these mutations is the truncation of the amino-terminal transactivation domain in the GATA1s protein making it transcriptionally less active than GATA1 (**Figure 1.4B**). The full length GATA1, and its shorter variant, GATA1s, are normally expressed at different levels in the mouse embryonic tissues (Calligaris *et al.*, 1995). This suggests that the balance between these proteins may be essential in normal development and in oncogenic transformation in the context of trisomy 21.

GATA1 mutations are unique to DS-AMKL and are not found in non-DS AMKL, in DS ALL or AMKL associated with myelodysplastic syndromes. Current data suggests that acquisition of *GATA1* mutations occurs *in utero*. This is supported by the findings of several groups who detected these mutations in DNA from neonatal blood spots. Importantly, almost all patients diagnosed with TMD contain

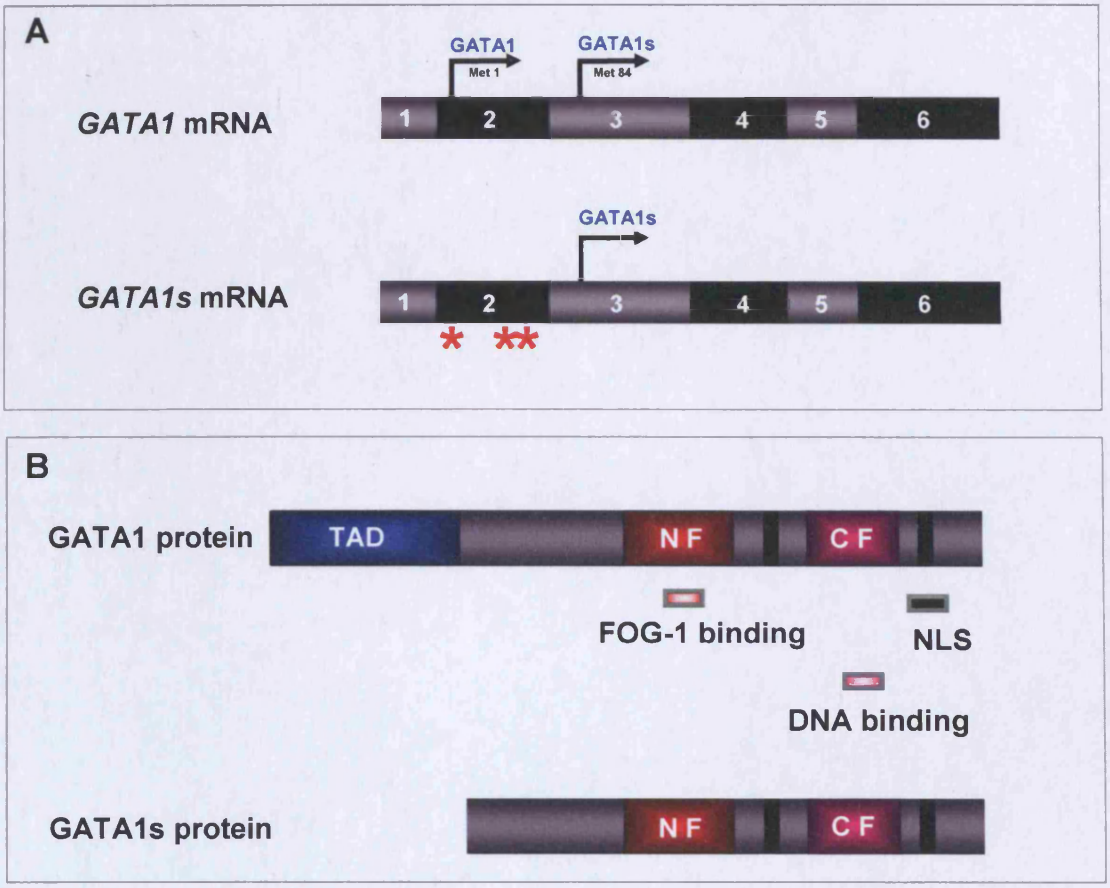


Figure 1.4. Diagram depicting the translation sites on *GATA1* and *GATA1s* mRNA and the functional domains of GATA1 and GATA1s proteins. A. The full length GATA1 is translated from the methionine 1 within exon 2. The first exon of *GATA1* is a non-coding exon. The shorter variant of GATA1, GATA1s is translated from the methionine 84 within exon 3. The GATA1s protein can be translated either from full length *GATA1* mRNA or from the *GATA1s* mRNA. *GATA1* mutations are found in DS individuals and almost all are located in the exon 2 as indicated by red asterisk. **B.** GATA1 is a 47-kDa protein containing a transactivation domain (TAD) at the amino-terminal region, two nuclear localisation sequences (NLS) and two zinc finger domains, one located at C-terminus (CF) which is essential for DNA binding. The N-terminal zinc finger (NF) is also required for DNA binding domain as well as interaction with FOG-1 protein. The GATA1s which is generated due to the use of an alternative translational start codon is a smaller 40-kDa protein that lacks the amino-terminal transactivation domain.

mutations in the *GATA1* gene. The study of DS patients showed that upon spontaneous resolution of TMD or AMKL remission, DS patients become negative for *GATA1* mutations (Rainis *et al.*, 2003; Pine *et al.*, 2005). Such mutations were also detected in DS cases where there was no clinical presentation of TMD or AMKL at birth (Ahmed *et al.*, 2004). Some studies, have failed to detect mutations in the *GATA1* gene at birth. This may be due to the limitation of techniques and material in Guthrie cards, which are currently used to identify these mutations which are thought to be present in rare clones.

1.6.3 The role of GATA1s in haematopoiesis

The functional importance of GATA1s has been demonstrated in several *in vitro* and *in vivo* studies. *GATA1* deletion mutants were generated to delineate the importance of different functional domains in inducing differentiation. Enforced expression of full length GATA1 induces megakaryocytic differentiation of the myeloid cell line 416B. The deletion of N-terminal transactivation domain, lost in GATA1s, did not affect megakaryocytic maturation of these cells (Visvader *et al.*, 1995). This suggests that GATA1s contains all domains essential for megakaryocytic differentiation.

The generation of a knock-in mouse containing GATA1s alone demonstrated the ability of this protein to maintain effective adult haematopoiesis. These mice exhibited normal megakaryopoiesis and erythropoiesis as measured by the number and morphology of platelets and red blood cells (Li *et al.*, 2005). In contrast, GATA1s caused aberrant foetal haematopoiesis. Foetal liver progenitors isolated from *GATA1s* mutant mice generated abnormal CFU-MK colonies *in vitro* and

there was a profound hyperproliferation of these progenitor cells when grown in liquid culture. This study suggests that GATA1s affects proliferation of foetal liver progenitor cells. In contrast to this animal study, in which adult haematopoiesis was unaffected, Hollanda *et al* demonstrated that the *GATA1s* mutation alone abrogates normal megakaryopoiesis and erythropoiesis in humans. Male individuals with the *GATA1s* mutation suffered from severe anaemia and had hyperproliferative and morphologically abnormal megakaryocytes (Hollanda *et al.*, 2006). Since *GATA1* is an X-linked gene, carrier females of the family harboured both normal *GATA1* and *GATA1s* mRNA. The mutation in the affected male individuals resulted in the production of GATA1s alone and the levels of GATA1s were the same as the levels in normal bone marrow. This study suggests that the N-terminal transactivation domain is required for normal erythropoiesis in humans and normal levels of GATA1s in the absence of GATA1 is not sufficient for effective haematopoiesis. The discrepancy in the role of GATA1s in animals and humans may be due to differences in the level of GATA1s protein expression, since this has been demonstrated to have an affect on haematopoiesis (Shimizu *et al.*, 2001). Shimizu *et al* generated transgenic mice expressing GATA1 with a deleted N-terminal domain. The potential of this transgene to rescue the *GATA1* mutant phenotype was investigated in the *GATA1.05* background. The levels of GATA1s transgene expression in comparison with the endogenous levels of GATA1 was a key factor in the efficiency of the rescue of the GATA1 phenotype. The increased levels of GATA1s were sufficient to rescue both primitive and definitive erythropoiesis, whereas similar expression levels to GATA1 led to inefficient definitive erythropoiesis.

1.7 ETS-Related-Gene (*ERG*)

ETS-relate gene (*ERG*) is a proto-oncogene located on chromosome 21 (21q22.3)(Rao *et al.*, 1987; Rao *et al.*, 1988). There are five isoforms of ERG produced as a result of differential mRNA splicing, alternative polyadenylation sites and translational codons (**Figure 1.5**). Both *ERG-2* and *ERG-3* mRNA expression is detectable in DS-AMKL patient samples and, in the megakaryoblastic cell lines, Meg-01 and CMK (Rainis *et al.*, 2005). All isoforms differ in their 5' regions and the expression of two exons. The A81 (81 bp) exon (ERS domain-*ERG* specific domain) is present in *ERG-1*, 2, 3 and $p38^{erg}$, whereas the A72 (72 bp) exon is present only in *ERG-3* (Prasad *et al.*, 1994).

ERG proteins can bind DNA via their Ets binding domain, which forms a Helix-loop-Helix structure (Schneikert *et al.*, 1992; Siddique *et al.*, 1993). In common with the RUNT domain, the ETS domain is involved in protein-protein interaction as well as DNA binding. Recombinant ERG proteins have been shown to be sequence specific transcritptional activators (Reddy and Rao, 1991). ERG can homodimerise and heterodimerise with Jun and the Fos/Jun AP1 complex via it ETS domain. The ERG isoforms can also form heterodimers with other member of the ETS family, such as FLI1, via the N-terminal pointed domain (Carrere *et al.*, 1998). There are now some studies suggesting a role for ERG in leukaemia and solid tumours. Baldus *et al* showed over-expression of ERG in AML patient samples. Since this was not always accompanied by genomic amplification, extra copies of the *ERG* gene are unlikely to explain the level of ERG expression in these samples (Baldus *et al.*, 2004). The over-expression of ERG in AML has been

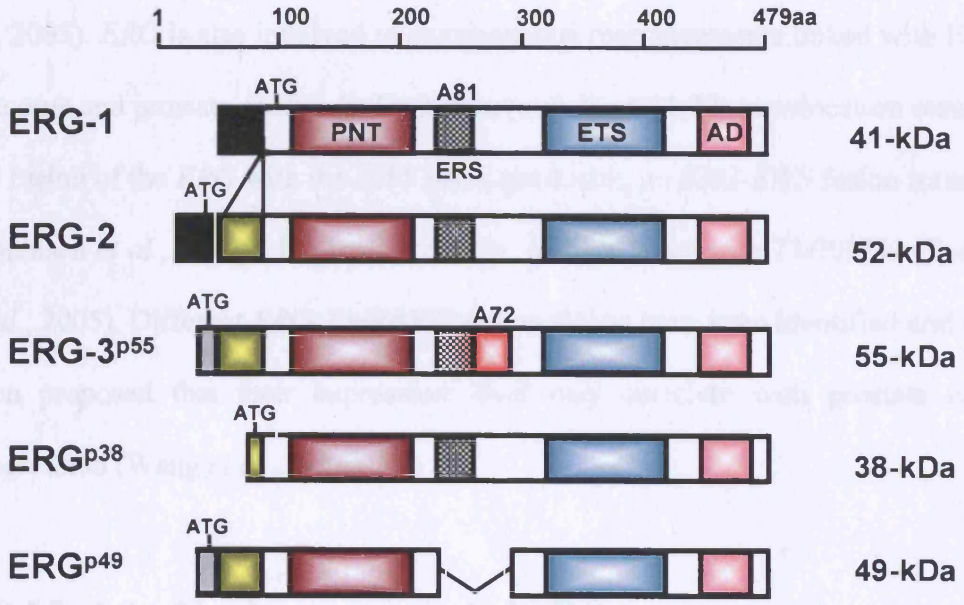


Figure 1.5. Schematic representation of different ERG isoforms. The five ERG isoforms are generated by differential mRNA splicing and alternative use of polyadenylation sites and translational codons. The functional domains include the pointed domain (PNT), located at the amino terminal region which is required for protein-protein interactions. The DNA binding, ETS domain and the activation domain (AD) are situated at the carboxyl terminal region. There are two exons, A81 (81 bp) or the ERG specific domain (ERS) and A72 (72 bp), which are not conserved in all the isoforms. A81 is present in ERG-1, 2, 3 and ERG^{p38}, whereas A72 (72 bp) exon which is only present in the ERG-3 isoform. These exons are rich in PEST sequences (P, proline; E, glutamic acid; S, serine; T, threonine). There is an inframe deletion of A72 and A81 in the ERG^{p49} with the 5' region being identical to the ERG-3 isoform. All isoforms also differ in their 5' region. The alternative parts in this region are represented by the yellow, grey and black boxes. Diagram adapted from Duterque-Coquillaud *et al.*, 1993.

reported by other groups where the levels of the protein have been closely correlated with the clinical outcome of AML with normal karyotype (Marcucci *et al.*, 2005). *ERG* is also involved in chromosomal rearrangements linked with Ewing Sarcoma and prostate cancer. In Ewing sarcoma the t(21;22) translocation results in the fusion of the *ERG* with the *EWS* gene, producing an *ERG-EWS* fusion transcript (Sorensen *et al.*, 1994). In prostate cancer, *ERG* is fused with *TMPRSS2* (Tomlins *et al.*, 2005). Different *ERG-TMPRSS2* fusion RNAs have been identified and it has been proposed that their expression level may correlate with prostate cancer progression (Wang *et al.*, 2006).

1.8 Model of leukaemogenesis in DS

The current model of leukaemogenesis in DS involves multiple steps with the first event being acquisition of trisomy 21. All the cells in an individual affected with DS contain trisomy 21, which occurs at the zygotic stage during development. A small percentage of patients develop TMD, which due to unknown mechanisms undergoes spontaneous remission. Somatic mutations in the *GATA1* gene are detected in TMD cases, which disappear upon remission. The result of these mutations is reduced megakaryocyte maturation and aberrant proliferation and accumulation of megakaryoblasts which are the hallmarks of AMKL in DS patients (**Figure 1.6**). These mutations are believed to occur at very early stages of development, since they are detected in almost all DS individuals that present with either TMD or AMKL.

It is becoming clear that *GATA1* mutations alone are not sufficient for leukaemic

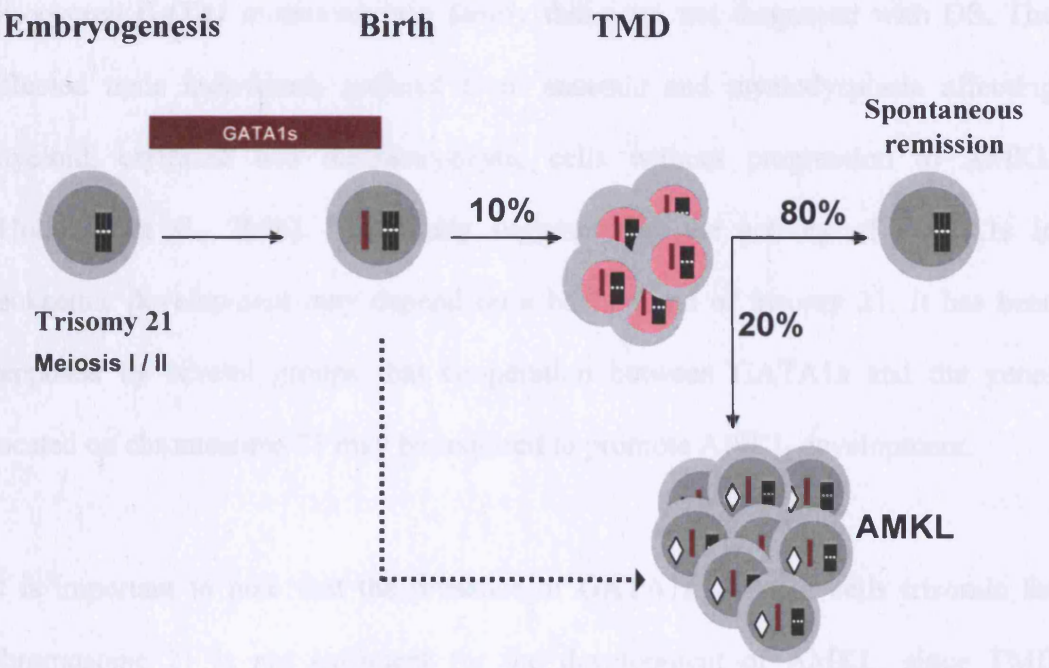


Figure 1.6. Model of leukaemogenesis in DS. It is generally believed that the development of leukaemia in DS is a multi-step process. The first event is the acquisition of an extra copy of chromosome 21, which is a distinguishing characteristic in DS. The origin of trisomy 21 is believed to be as a result of homologous chromosome non-disjunction at meiosis stage I and rarely at meiosis stage II, which occurs during embryonic development. The second step is believed to be somatic mutations in the *GATA1* gene which creates a shorter product, *GATA1s*. This mutation is detected in almost all DS patients at birth and thus is predicted to occur at very early stages during development. Approximately 10% of individuals with DS develop transient myeloproliferative disorder (TMD), most of which undergo spontaneous remission. *GATA1* mutations are lost in TMD patients in remission. Around 20% of TMD patients will develop acute megakaryoblastic leukaemia (AMKL). DS patients as a whole have a 600 fold greater chance of developing AMKL than non-DS patients. It is predicted that the *GATA1s* mutation provides the pro-proliferation signal required for the accumulation of megakaryoblasts detected in AMKL. However, these mutations are not sufficient for leukemic development. Genes located on chromosome 21 and other as yet unidentified mutations (diamond) may provide the necessary events for the progression of TMD to AMKL and the development of leukaemia in DS.

development or progression of TMD to AMKL in DS individuals. Hollanda *et al* discovered *GATA1* mutations in a family that were not diagnosed with DS. The affected male individuals suffered from anaemia and myelodysplasia affecting myeloid, erythroid and megakaryocytic cells without progression to AMKL (Hollanda et al., 2006). This study suggests that the activity of GATA1s in leukaemic development may depend on a background of trisomy 21. It has been proposed by several groups that cooperation between GATA1s and the genes located on chromosome 21 may be required to promote AMKL development.

It is important to note that the presence of GATA1s alone in cells trisomic for chromosome 21 is not sufficient for the development of AMKL, since TMD disappears in the majority of cases. It has recently been demonstrated that GATA1s affects progenitor cells at a specific developmental stage (Li et al., 2005). It has been proposed that, in the setting of trisomy 21, additional mutations are necessary at this particular point in development for leukaemic progression of TMD clones. In this context, Ahmed *et al* detected *GATA1* mutations in DS individuals that did not present with AMKL, further suggesting that additional factors other than *GATA1* mutations and genes located on trisomy 21 are required for leukaemic transformation in DS patients (Ahmed et al., 2004).

In summary, *GATA1s* and genes located on chromosome 21 are required but not sufficient for AMKL development in DS. It is proposed that overexpression of genes on chromosome 21 may promote megakaryopoiesis and increase the pool of early megakaryocytic progenitors, while the acquired *GATA1* mutation increases proliferation of these megakaryoblasts at the expense of differentiation.

1.9 Myelodysplastic syndrome (MDS)

In addition to TMD, some DS patients are predisposed to childhood myelodysplastic syndrome (MDS) prior to AMKL development. MDS precedes AML in approximately half of cases in DS (Zipursky *et al.*, 1992; Creutzig *et al.*, 1996; Lange *et al.*, 1998). DS patients can take several months and up to few years to progress from MDS to AML (Creutzig et al., 1996). Myelodysplatic syndromes are a group of clonal haematopoietic disorders, characterised by aberrant proliferation and differentiation of the myeloid lineage. The bone marrow is normo or hypercellular and ineffective haematopoiesis causes severe peripheral cytopenia and trilineage dysplasia (abnormal cellular morphology) involving myeloid, erythroid and megakaryocyte cells. The dysplastic characteristics include micromegakaryocytes, hypersegmented neutrophils and ringed sideroblasts. DS patients with MDS have ineffective thrombopoiesis and exhibit dysplastic changes in both erythroid and megakaryocytic progenitors. The ineffective haematopoiesis has been suggested to be a result of high levels of apoptosis and proliferation in the bone marrow. Thus, Albitar and *et al* have detected increased levels of apoptosis in MDS patients, as measured by Annexin V staining and levels of caspase-3 activity (Albitar *et al.*, 2002). As the disease progresses, the bone marrow cells become more abnormal and in 30-40% of MDS cases, patients develop acute myeloid leukaemia (AML) (Matsushima *et al.*, 2003). Evolution to AML, infection and bleeding are the main cause of death in MDS patients.

MDS is primarily a disease of elderly people with the median age at diagnosis being between 65 and 75 years. In addition, mylodysplastic syndromes account for

about 2% of haematological malignancies in children. There are five subtypes of MDS as classified by the French-American-British (FAB) study group. The different subtypes are based on the percentage of blasts in the bone marrow (**Table 3**) and all indicators of good prognosis are associated with particular sub-types of MDS. Although widely used, this type of classification does not take cytogenetic abnormalities and genetic changes that occur in MDS into consideration.

Table 3: Subtypes of MDS as classified by FAB study group.

FAB subtype	**% of blasts**
Refractory anemia (RA)	**< 5%**
Refractory anemia with ringed sideroblasts (RARS)	<5%
Refractory anemia with excess blasts (RAEB)	**6-20%**
Refractory anemia with excess blasts in transformation (RAEB-T)	20-30%
Chronic myelomonocytic leukaemia (CMML)	**As any of above with or without increase of monocytes**

Several features distinguish MDS from *de novo* AML. There are distinct differences in the haematopoeitic cells detected in MDS patients, as compared with AML. In MDS, there is lack of terminally differentiated cells or cells have dysplastic morphology. In contrast, differentiation is blocked in AML and cells have a blastic morphology. In addition, there is an increased risk of apoptosis at early stages of MDS and the disease is highly associated with abnormalities of chromosme 7 and 5. However, the most common chromosomal translocations

associated with AML are inv (16) a t(16;16), t(15;17) and t(8;21). Furthermore, in contrast to AML, MDS patients respond poorly to treatment with cytosine arabinoside.

1.9.1 Murine models of MDS

There are currently several murine models of MDS that suggest the potential importance of different genes in the pathogenesis of MDS and AML evolution. *EVI1* is involved in chromosomal translocation (t(3;3)(q21;26) and inv(3)(q21q26)) associated with MDS. Mice injected with BM-derived progenitor cells transduced with *EVI1* expressing retrovirus display some phenotypic characteristics of MDS (Buonamici *et al.*, 2003). Megakaryocytic and erythroid hyperplasia was detected in the BM and these cells had reduced expression levels of EPO and TPO receptors, which are required for effective differentiation.

Transgenic mice expressing the *NUP98-HOXD13* fusion gene are also defective in myeloid differentiation and develop MDS and eventually AML (Lin *et al.*, 2005). Anaemia and increased apoptosis, features exhibited in MDS patients, were detected in these mice. In a recent study, retroviral insertional mutagenesis revealed the collaborative role of *Erg*, *Meis1*, *Mn1* and miRNA genes with *NUP98-HOXD13* in inducing leukaemic transformation (Slape *et al.*, 2007). This mouse model did not completely recapitulate the features observed in patients with MDS, since some of the mice developed ALL, a leukaemia which is rarely detected in these patients (Disperati *et al.*, 2006). Another murine model of MDS with an AML evolution was demonstrated by generation of a transgenic mouse expressing *BCL2* and a *RAS*

mutation (*NRASD12*), where *BCL2* is expressed in a conditional tetracycline-inducible manner (Omidvar *et al.*, 2007). This mouse line was generated by crossing the conditional *BCL2* line with the constitutive *NRAS* mutation. Conditional expression of BCL2 was shown to co-operate with constitutive NRAS expression to induce high risk MDS with increased apoptosis of haematopoietic cells. BCL2 was shown to be an initiating factor in inducing MDS and it was also required for the maintenance of the disease. In contrast, the constitutive expression of both BCL2 and NRASD12 (expression driven by myeloid MRP8 promoter) resulted in AML with no apoptotic cells. This study shows that two cooperating events, lead to MDS development and progression to AML in mice. It was proposed that the differences between the two models may be the type of leukaemic cell targeted by BCL2. In the MDS model, the *tTABCL2* is under the control of the MMTV promoter, which drives the inducible expression of BCL2 in different cell types as compared to the AML model, where the constitutive expression of BCl2 is targeted to myeloid progenitors by the MRP8 promoter.

A recent report has highlighted the possible contribution of RUNX1 to MDS pathogenesis. *RUNX1* mutations are correlated with MDS patients that progress to AML. Recipient mice transplanted with BM cells transduced with *RUNX1* mutants (mutations in the RUNT domain) succumbed to an MDS like disorder as characterised by peripheral cytopenia and erythroid dysplasia (Watanabe-Okochi *et al.*, 2008).

Finally, mice heterozygous for *NPM,* exhibit most features detected in human MDS, including erythroid dysplasia, defective megakaryopoiesis and bone marrow

hyperplasia (Grisendi *et al.*, 2005). The percentage of Ter119 positive cells was markedly increased in the bone marrow of $NPM^{+/-}$ as compared with NPM wild type mice. The effect of *NPM* heterozygosity on the accumulation of immature erythroblasts was shown by the increase in $Ter119^{hi}CD71^{hi}$ expressing cells. Although these mice displayed dysplastic features in the megakaryocyte-erythrocyte lineage, there were no observed abnormalities in the macrophages or granulocytes. In addition, *NPM* heterozygotes were shown to have increased risk of developing haematological malignancies compared with *NPM* wild type mice (Sportoletti *et al.*, 2008).

1.9.2 Chromosomal abnormalities associated with MDS

There are several different chromosomal abnormalities associated with MDS. These include monosomy and deletions of chromosome 5 and 7, trisomy 8, 20q deletion, loss of chromosome Y and inv(3) (inversion of chromosome 3). Trisomy 8 is the most common cytogenetic abnormality correlated with MDS and it is accompanied with poor prognosis and short survival (Pedersen, 1997). Various chromosomal translocations have also been linked to MDS. Some of the genes involved include *EVI1*, *TEL* (*ETV6*), *MLL*, and Nucleoporin (Mitani *et al.*, 1994; Wlodarska *et al.*, 1995; Taki *et al.*, 1997; Ahuja *et al.*, 1999; Grisendi et al., 2005). In addition, the t(3;5)(q25.1;q34) rearrangement results in a fusion transcript comprised of the nucleophosmin (*NPM*) gene and the myeloid leukaemia factor 1 (*MLF1*) gene (Yoneda-Kato *et al.*, 1996). Existence of more than one type of abnormal karyotype is more common in therapy related MDS (t-MDS) than in *de novo* MDS. No clear correlation has been found between any chromosomal aberrations or specific gene with the onset or progression of MDS.

1.9.3 Nucleophosmin (NPM)

NPM (also known as B23, numartin or NO38) is a ubiquitously expressed phosphoprotein that has a major role in ribosomal assembly and biogenesis. NPM is predominantly localised in nucleoli, although it can shuttle between the cytoplasm and nucleus. The functional motifs of NPM are well characterised. At the N-terminus, the protein has a non-polar domain and two highly acidic segments. Both regions are required for the chaperone function of NPM as revealed by deletion and functional (Szebeni and Olson, 1999) studies. NPM contains a bipartite nuclear localisation signal (NLS) that has been shown to associate with several proteins such as the HIV (Human Immunodeficiency Virus) rev protein (Fankhauser *et al.*, 1991). The C-terminal section of the protein is basic, apart from the last 35 residues, which forms an aromatic rich region. These domains are involved in nucleic acid binding. The C-terminal portion of NPM in combination with the acidic region is also required for the ribonuclease activity of the protein (Hingorani *et al.*, 2000) (**Figure 1.7A**).

There are several reports suggesting an important role for NPM in cell proliferation. NPM has been shown to inhibit the transcriptional activity of several tumour suppressor proteins including IRF1 (interferon regulatory factor 1) and PKR (interferon (IFN)-inducible, double-stranded RNA-dependent protein kinase). NPM induces IRF1 inactivation by interacting with the protein and interfering with its DNA binding (Kondo *et al.*, 1997). Indeed, IRF1 inactivation as a result of exon skipping, which causes removal of the DNA binding domain, is detected in 30% of MDS patients (Harada *et al.*, 1994). In addition, NPM induction of cellular

proliferation is partly mediated by interaction with and inhibition of the catalytic activity of PKR (Pang *et al.*, 2001). PKR induces apoptosis and prevents cell growth by inhibiting mRNA translation and its activation is associated with the pathogenesis of several diseases such as Fanconi anaemia (FA) (Pang et al., 2001). Overexpression of NPM was shown to inhibit PKR-mediated apoptosis in FA derived cell line (Pang *et al.*, 2003). The high expression level of NPM in leukaemia and leukaemic cell lines further supports a role for NPM in cell growth and survival (Kondo et al., 1997). The activity of NPM in these processes is likely to be complex. Thus, mouse fibroblasts overexpressing NPM become resistant to apoptosis induced by UV damage (Wu and Yung, 2002). In contrast, NPM has been shown to enhance and activate the transcriptional activity and function of the tumour suppressor protein p53 (Colombo *et al.*, 2002; Kurki *et al.*, 2004; Korgaonkar et al., 2005).

NPM also has a role in maintaining genomic stability, by regulating centrosome duplication during cell cycle (Okuda *et al.*, 2000). To ensure the correct number of chromosomes are acquired by daughter cells, centrosome duplication occurs only once per cell cycle. This process is tightly regulated by proteins such PML (Xu *et al.*, 2005), CDK2/cyclinE kinase and NPM. At early G1 phase of the cell cycle, NPM is bound to the centrosomes. The dissociation of centrosomes from NPM is required for centrosome duplication and this process is triggered upon phosphorylation of NPM by CDK2/CyclinE complex. After this, NPM re-associates with centrosomes during mitosis. Aberrant regulation of centrosome duplication has been correlated with abnormal chromosome segregation and aneuploidy in AML patients (Neben *et al.*, 2004). *NPM* mutations, which are highly

associated with AML may account for this deregulation of centrosome duplication.

Recent evidence has highlighted the importance of NPM in tumourgenesis. The NPM locus is involved in chromosomal translocations associated with several human malignancies (Morris *et al.*, 1994; Redner *et al.*, 1996). In addition, NPM is commonly mutated in blasts of AML patients with normal karyotype (Falini *et al.*, 2005; Nakagawa *et al.*, 2005). The mutations occur in exon 12 of the NPM gene. These frameshift mutations truncate the C-terminal region, causing loss of at least one of W288 and W290 tryptophan residues necessary for nuclear localisation. In addition they cause a gain of an NES at the C-terminus, which results in aberrant localisation of NPM in the cytoplasm ($NPMc^+$) (Bolli *et al.*, 2007; Falini *et al.*, 2007) (**Figure 1.7B**). AML patients with $NPMc^+$ have a much better responsiveness to chemotherapy when compared with patients lacking this mutation. $NPMc^+$ is frequently correlated with *CEBPA* and *NRAS* mutations and internal tandem duplications of *FLT3* (FLT3/ITD) (Falini *et al.*, 2006). These patients appear to have a worse prognosis compared with patients harbouring $NPMc^+$ alone (Christensen and Weissman, 2001).

1.9.4 Myeloid Leukaemia Factor 1 (MLF1)

MLF1 was first identified as a gene fused with *NPM* in the t(3;5) translocation associated with MDS. MLF1 does not belong to any previously identified protein family and it comprises of a 14-3-3 interaction motif at its N-terminus and an adaptor molecule (Madm, MLF1 adaptor molecule) binding domain (Williams *et al.*, 1999). MLF1 contains both a classic RSXSXP 14-3-3 binding motif and a non-consensus RXSXSX motif.

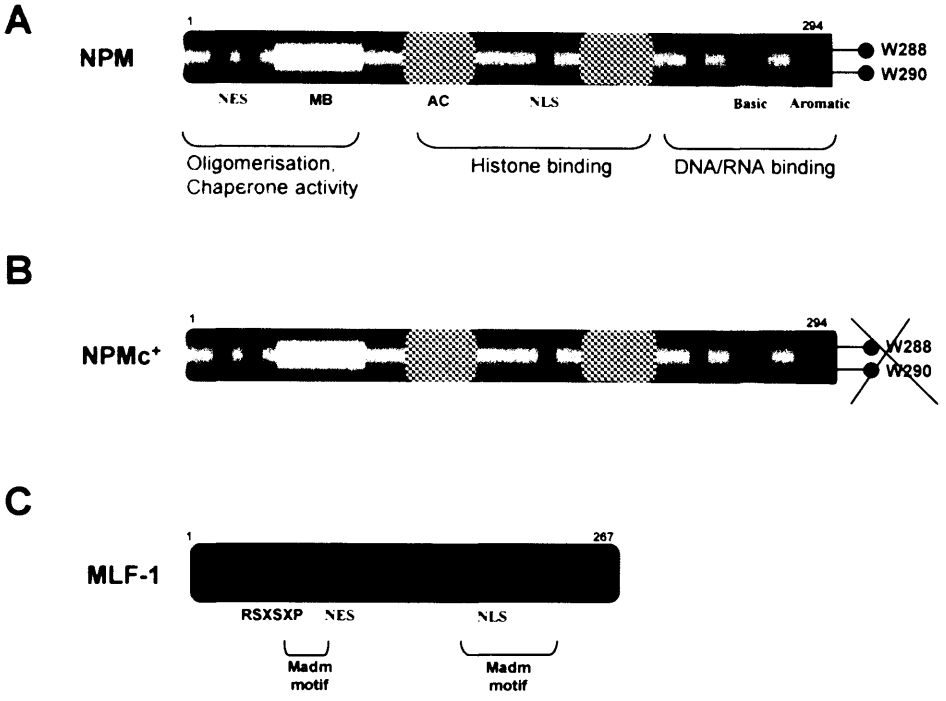


Figure 1.7. Schematic representation of NPM, cytoplasmic NPM and MLF1. A. NPM is a 249 amino acid protein comprising two nuclear export signal (NES) motif at its N-terminus, a metal binding domain (MB), two acidic amino acid clusters (AC) and two nuclear localisation signals (NLS). There is a basic region and an aromatic region at the C-terminus. NPM contains two tryptophan residues 288 and 290 at the C-terminus required for nuclear localisation. The N-terminus region is required for oligomerisation and chaperone activity of NPM, whereas the C-terminus is involved in DNA or RNA binding. An internal segment of NPM protein is involved in histone binding. **B.** Frameshift mutations lead to loss of the tryptophan residues (most commonly W290) required for nuclear localisation and result in a gain of NES at the C-terminus. Both of these are required for aberrant localisation of NPM in the cytoplasm ($NPMc^+$). **C.** MLF1 is a 268 amino acid protein containing two motifs required for interaction with Madm protein and two putative RSXSXP motifs required for 14-3-3 binding. MLF1 contains NES and two NLS domains.

Recently, an NES and two NLS motifs have been identified in MLF1 (Winteringham *et al.*, 2006; Yoneda-Kato and Kato, 2008) (**Figure 1.7C**). MLF1 is expressed in a tissue specific manner and its localisation in the cytoplasm is dependent on its interaction with 14-3-3 family member, 14-3-3-ζ protein. MLF1 interaction with MADM results in the recruitment of a serine kinase that leads to the phosphorylation of MLF1 (Lim *et al.*, 2002). The 14-3-3-ζ protein interacts with the phosphorylated MLF1 keeping the protein within the cytoplasmic compartment of a cell (Winteringham et al., 2006).

The murine homolog of *MLF1*, *Hls-7* (haematopoietic lineage switch 7), is involved in erythroid/myeloid lineage switching (Williams et al., 1999). HLS-7 has 79% amino acid identity and a 90% homology (includes amino acids that are different but do not affect the structure of the protein) with human MLF1. HLS7 prevents erythropoietin (EPO) dependent differentiation of J2E erythroleukemic cells, which are immortalised at the proerythroblast stage. In contrast, ectopic expression of the protein promotes myeloid differentiation of M1 monoblasts. HLS7 mediates a block of erythroid differentiation by suppressing the upregulation of the CDK2 inhibitor, p27, and preventing cell cycle arrest (Winteringham *et al.*, 2004).

In common with hMLF1, *Drosophila* MLF1 (*dMlf1*) contains a 14-3-3 interaction motif and it is localised in the cytoplasm. It has been suggested that dMLF1 can translocate into the nucleus and associate with the transcription factor, DREF (DNA replication related element-binding factor), which regulates several genes required for proliferation in *Drosophila* (Ohno *et al.*, 2000).

1.9.5 NPM-MLF1

In the t(3;5) (q25;q34) translocation, the *NPM* gene located on chromosome 5 is fused with *MLF1* gene on chromosome 3, creating a *NPM-MLF1* fusion gene (**Figure 1.8**). The C-terminal region of NPM, containing one NLS and part of the acidic region, is truncated as a result of the translocation and NPM is fused with MLF1, lacking first 15 residues at the N-terminal end. The NPM-MLF1 protein is predominantly localised to the nucleus. Despite the fact that the MLF1 portion of the NPM-MLF1 protein is phosphorylated in the cytoplasm of cells, it does not interact with the 14-3-3-ζ protein. However, due to the presence of the NLS within the NPM moiety of NPM-MLF1, the fusion protein can translocate into the nucleus (Lim et al., 2002).

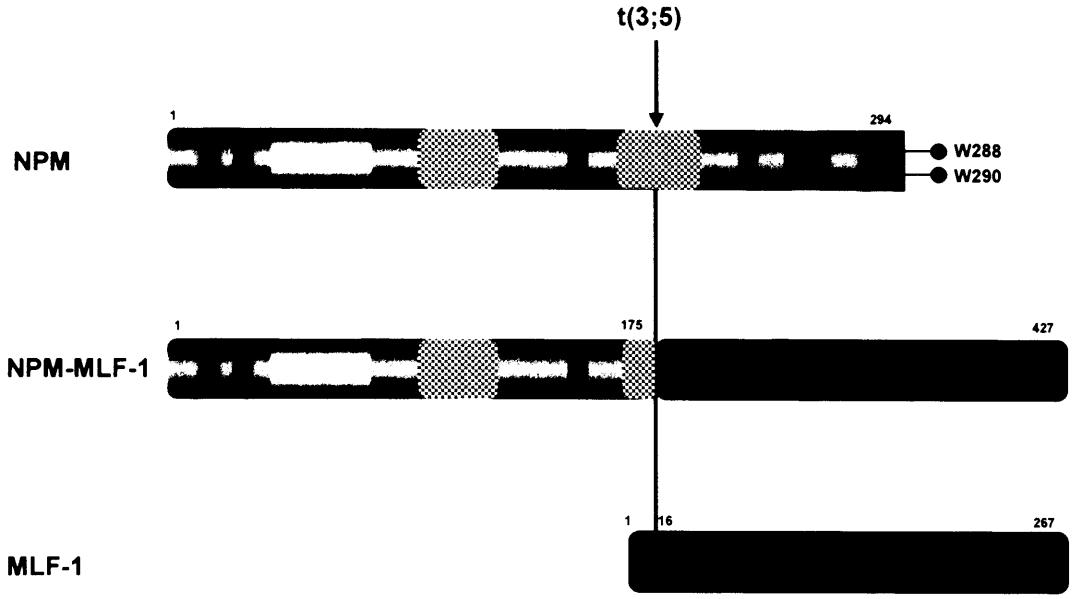


Figure 1.8. Schematic representation of NPM-MLF1. The arrow indicates the point at which the NPM and MLF1 proteins are fused. The acidic domain and one NLS are truncated due to the translocation resulting in the fusion of the N-terminus portion with almost the entire MLF1, lacking the first 15 residues. This creates a fusion protein of 428 amino acids.

Project aims

The main aim of this study was to analyse the molecular basis for haematological abnormalities leading to MDS and AMKL. This was investigated using two models. In a DS associated AMKL model, the potential synergy between the GATA1 mutation and a chromosome 21 gene, *ERG-3*, in the development of AMKL was investigated. The second model is t(3:5) associated with MDS, and since t(3;5) is the most common translocation, the potential role of the resultant NPM-MLF1 fusion protein in the development of MDS was investigated.

Chapter 2

Materials and Methods

2.0 Generation of retroviral constructs

Retroviral vectors used in this study include the pMSCV-neo and pMSCV-IRES-hCD2 vector. Both vectors contain the 5' LTR (long term repeat) and 3' LTR, Ψ^+ (viral packaging signal) and Amp^r (ampicillin resistance gene). The pMSCV-neo comprises the murine PGK (phosphoglycerate kinase) promoter which controls the expression of Neo^r (neomycin resistance gene). The pMSCV-IRES-hCD2 contains IRES (Internal-ribosomal-entry-site) and hCD2 (human CD2 tailless gene).

2.1 pMSCV-*ERG-3*-IRES-CD2

The pMSCV-IRES-CD2 retroviral vector was digested with the *EcoRI* enzyme. The *ERG-3* cDNA is flanked by *MfeI* sites. The *ERG-3* cDNA was cloned into the *EcoRI* sites in the pMSCV vector upstream of the IRES and the gene encoding hCD2. The fragment was confirmed by digestion of the vector with *BamHI*, which releases the *ERG-3* insert fragment of 1887 bp.

2.1.1 pMSCV-neo-FLAG-*Gata1*

A CMV expression vector containing *Gata1* cDNA (1241 bp) cloned as a *BamHI* and *XhoI* insert was obtained from Dr Izraeli (Sheba Medical Center, Israel). There is a sequence for the FLAG epitope tag at the N-terminus of *Gata1* sequence. The FLAG-*Gata1* fragment was digested with *SacI* and overhangs were filled in with Klenow to create a blunt 5′ end. The fragment was released by cutting with *XhoI*. The pMSCV-neo plasmid was digested with blunt enzyme *HpaI* and *XhoI*. The *SacI*-FLAG-*Gata1*-*XhoI* fragment was ligated into the *HpaI* and *XhoI* sites in the

pMSCV vector.

2.1.2 pMSCV-neo-FLAG-*Gata1s*

The CMV expression vector containing the *Gata1s* cDNA (1021 bp) cloned in *EcoRI* sites was obtained from Dr Izraeli. The pMSCV-neo vector was digested with *EcoRI* and Phophatase treated to prevent re-ligation of the vector. The FLAG-*Gata1s* cDNA was released from the CMV vector by digesting with *EcoRI.* This fragment was subsequently cloned into the *EcoRI* sites of the pMSCV-neo vector upstream of PGK-Neo^r. The orientation of the insert was determined by restriction digest and sequencing.

2.1.3 pMSCV-neo-FLAG-*NPM-MLF1*

The pCDNA3 expression vector containing the *NPM-MLF1* cDNA (1026 bp), cloned as a *HindIII* and *XhoI* insert, was obtained from Stephen Morris (St. Jude Children's Research Hospital). The pCDNA3-*NPM-MLF1* cDNA was used as a template in a PCR reaction. Primers were designed to amplify the *NPM-MLF1* cDNA by integrating *EcoRI* sites at either end of the *NPM-MLF1* cDNA, as well as adding an 5′ Flag epitope sequence. The pMSCV-neo vector was digested with *EcoRI* and phosphatase treated to prevent re-ligation of the vector. The amplified *NPM-MLF1* cDNA was digested with *EcoRI*, purified and cloned into the pMSCV-Neo vector, upstream of PGK-Neo^r. The orientation of the insert was determined by restriction digest and sequencing.

2.1.4 pMSCV-neo-FLAG-*NPM*

The CMV expression vector containing the *NPM* cDNA (984 bp) was obtained from Dr Colombo (Colombo *et al.*, 2002). PCR primers were designed to amplify the cDNA by integrating *EcoRI* sites at either end of the *NPM* cDNA, and adding a FLAG epitope sequence at the 5′ end. The pMSCV-neo vector was digested with *EcoRI* and phosphatase treated to prevent re-ligation of the vector. The amplified *NPM* cDNA was digested with *EcoRI*, purified and subsequently cloned into MSCV-Neo vector upstream of PGK-Neo^r. The orientation of the insert was determined by restriction digest and sequencing.

2.1.5 pMSCV-neo-*tMLF1*

The *MLF1* moiety of *NPM-MLF1* was generated using the *NPM-MLF1* pMSCV-neo construct as a template. The following primers were used for PCR amplification:

Forward primer: 5′-AAAGAATTCACCATGGACTACAAGGACGACGACAA GAGTCCATTCTTGCACACCGA-3′

Reverse primer (*MLF1* Reverse: M-R): 5′-AAACTCGAGTTATTTTTTGTTGC TTTTCACAGATGA-3′

The pMSCV-neo vector was digested with *EcoRI* and *XhoI* restriction enzymes. The t*MLF1* (truncated MLF1) PCR product (768 bp) was digested with *EcoRI* and *XhoI*, purified and cloned into the pMSCV-Neo vector upstream of PGK-Neo^r.

2.1.6 pMSCV-neo-*MLF1*-myc

Full length *MLF1* was generated using the pMSCV-neo-t*MLF1* construct as a

template. The following primers were used for PCR amplification:

Forward primer: 5′-AAAGAATTCACCATGTTCAGGATGCTGAACAGCAGT

TTTGAGGATGACCCCTTCTTCTCTCATTCTTGCACACCGA-3′

Reverse primer: 5′-AAACTCGAGTTACAGATCTTCTTCAGAAATAAGTTTTT

GTTCTTTTTTGTTGCTTTTCACAGATGA-3′

The pMSCV-neo vector was digested with *EcoRI* and *XhoI* restriction enzymes. The *MLF1* PCR product (806 bp) was digested with with *EcoRI* and *XhoI*, purified and cloned into pMSCV-Neo vector upstream of PGK-Neo^r.

2.1.7 pMSCV-neo-FLAG- *MLF1*-NLS

The *MLF1* moiety of *NPM-MLF1* containing *NPM* derived nuclear localization signal at the N-terminus was generated using the pMSCV-neo-*NPM-MLF1* construct as a template. The following Forward primer was used for PCR amplification and M-R (shown above) was used as a reverse primer.

Forward primer: 5′-AAAGAATTCACCATGGACTACAAGGACGACGACAA

AAGAGACTTCCTCCACTG-3′

The pMSCV-neo vector was digested with *EcoRI* and *XhoI* restriction enzymes. The PCR product was digested with *EcoRI* and *XhoI*, purified and cloned into pMSCV-Neo vector upstream of PGK-Neo^r.

2.1.8 pMSCV-neo-FLAG-*tNPM*

The *NPM* moiety of *NPM-MLF1* was generated by using the pMSCV-neo-FLAG-*NPM-MLF1* construct as a template. The following primers were used for PCR amplification (*NPM* primer sequence obtained from Bertwistle *et al.*, 2003). The

Forward primer contains N-terminal FLAG tag coding sequences.

Forward primer (*NPM* Forward: N-F): 5′-AAAGAATTCACCATGGACTACAAG GACGACGACAAGATGATGATGATGATTTTGATGAT-3′

Reverse Primer: 5′-AAACTCGAGTTAAAGAGACTTCCTCCACTGCCA-3′

The pMSCV-neo vector was digested with *EcoRI* and *XhoI* restriction enzymes. The PCR product was digested with *EcoRI* and *XhoI*, purified and cloned into pMSCV-Neo vector upstream of PGK-Neo^r.

2.1.9 pMSCV-neo-FLAG-*NPM*ΔNLS

The *NPM* retroviral construct lacking the nuclear localization signal was generated using the pMSCV-neo-*NPM* construct as a template. The following primers were used for PCR amplification (*NPM* primer sequence obtained from Bertwistle *et al.*, 2003). The forward primer N-F was used for amplification of tNPM was used (shown above).

Reverse primer: 5′-AAACTCGAGTTACACAGCTACTAAGTGCTG-3′

The pMSCV-neo vector was digested with *EcoRI* and *XhoI* restriction enzymes. The PCR product was digested with *EcoRI* and *XhoI*, purified and cloned into MSCV-Neo vector upstream of PGK-Neo^r.

2.2 Sequencing primers for pMSCV retroviral vectors

The primers used for sequencing of the pMSCV-neo vector include 5′-CCCTTGAACCTCCTCGTTCGACC, and 3′-GAGACGTGCTACTTCCATTTGT

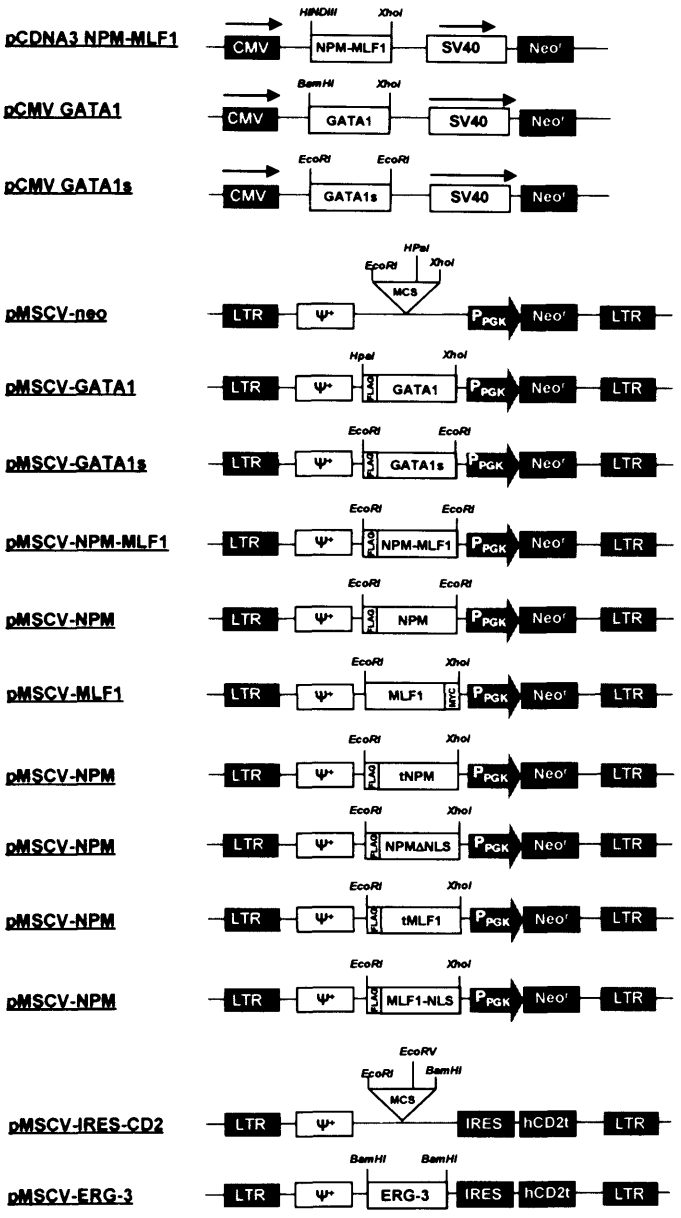


Figure 2.1. A diagram of the retroviral constructs used in this study. LTR: long term repeat, IRES- (internal ribosome entry site), Neo^r: neomycin resistance gene, P_{PGK}: phosphoglycerate kinase promoter, MSC: multiple cloning site, hCD2t: human CD2 tail-less gene, ψ^+: viral packaging signal, SV40: Simian Virus 40 early promoter, CMV: human cytomegalovirus promoter.

C, and a 3'- CACATTGCCAAAAGACGG primer was used for the pMSCV-hCD2 vector.

2.3 Cell lines

The human megakaryoblastic cell line, Meg-01 (ATCC), was maintained in RPMI (Roswell Park Memorial Institute) containing 10% FCS, 2 mM L-Glutamine, 100 U/mL Penicillin, 100 μg/mL Streptomycin (Gibco), 10 mM Hepes, 1 mM sodium pyruvate, 4.5 g/L glucose and 1.5 g/L sodium bicarbonate. The human pro-erythroblastic cell line, K562 (ATCC), were maintained in RPMI containing 10% FCS, 2 mM L-Glutamine and 100 μg/mL streptomycin and 100 units/mL (U/mL) penicillin (Gibco). Mouse NIH3T3 fibroblasts and LinXE ecotropic packaging cells were cultured in complete DMEM (Dulbeccos Modified Eagle Media) (Gibco) medium containing 10% FCS, 2 mM L-Glutamine, 100 μg/mL Streptomycin and 100 U/mL Penicillin (Gibco). The LinXE cells (Genetica) (Hannon *et al.*, 1999) were maintained in 75 μg/mL hygromycin B (Calbiochem) to select for the presence of env (envelope), gag (group antigen) and pol (reverse transcriptase) genes.

2.4 Transfection of LinXE cells

LinXE cells were transfected with 8 μg DNA. Lipofectamine reagent (Invitrogen) was used at a 5:1 ratio of Lipofectamine:DNA. The Lipofectamine-DNA mixture was added to a final volume of 1.6 mL of Optimem medium (GIBCO). The mixture was incubated at room temperature (RT) for 30 min. Adherent LinXE cells were washed with optimem and 6.4 mL of optimum was added to the cells. After 30 min

the Lipofectamine-DNA mixture was added to the cells and incubated at 37°C for 5 hrs after which 8 mL of DMEM media containing 20% FCS was added to the cells. The following day, media was replaced with fresh DMEM media containing 10% FCS. The viral supernatant was collected 48 hrs after transfection and used to transduce haematopoietic progenitor cells (HPCs).

2.5 Isolation of haematopoietic progenitor cells (HPCs)

Foetal liver cells were harvested from day 12 embryos. Single cell suspensions were prepared. Monoclonal antibodies specific to c-Kit (2B8) (Pharmingen) and Ter119 (BD Biosciences) was used to purify progenitor cells. The purification was carried out using a magnetic activated cell sorter (MACS) (Miltenyi Biotech) according to the manufacturer's instructions. For adult progenitor cell purification, bone marrow was extracted from 5-Fluorouracil (5-FU) treated mice. Red cells were lysed by resuspending cells in 1 M Tris (pH 7.2) and 0.144 M NH_4Cl for 10 min at room temperature. Isolated HPCs were cultured overnight in DMEM containing 10% FCS, 100 ng/mL SCF, 10 ng/mL IL-3, 10 ng/mL IL-6 (Peprotech-EC, London, United Kingdom) and 50 μM 2-mercaptoethanol.

2.6 Transduction of HPCs

Retroviral supernatant was produced by transfecting the LinXE ecotropic retrovirus packaging cell line with retroviral vectors. Supernatant was harvested after 48 hours and cleared of cell debris by centrifugation at 580g for 5 mins. Virus was aliquotted into 1.5 mL eppendorf tubes and concentrated for 1 hour at 16,000g. The supernatant was discarded and the virus was concentrated 8 fold. The viral

supernatant was used to transduce the purified c-Kit$^+$Ter119$^-$ HPCs by spinoculation (centrifugation at 700g, 25°C, 45 minutes) in the presence of 100 ng/mL SCF, 10 ng/mL IL-3, 10 ng/mL IL-6 and 5 μg/mL polybrene (Sigma-Aldrich, Poole, United Kingdom). Transduced HPCs were cultured with the same cytokine conditions for 24 hours and subsequently used for colony forming assays.

2.7 Colony forming assays

2.7.1 Myeloid conditions

Transduced progenitor cells were cultured at 10^4/mL in methylcellulose medium 3434 (Stem Cell Technologies), supplemented with 10 ng/mL GM-CSF and 1 mg/mL of G418 (Gibco) in the first round of plating. Cells were plated without selection in the subsequent rounds of plating. Cells were incubated at 37°C for 7 days after which colonies were counted and cells were harvested and analysed by flow cytometry.

2.7.2 Megakaryocytic conditions

Transduced progenitor cells were cultured at 10^4/mL in methylcellulose media 3234 (Stem Cell Technologies) in the presence of 50 ng/mL TPO (R&D systems), 50 ng/mL SCF and 1 mg/mL G418 (Gibco) in the first round of plating. Cells were plated without selection in the subsequent round of platings. Cells were incubated at 37°C for 5-10 days after which colonies were counted and cells were harvested and analysed by flow cytometry.

2.8 Generation and maintenance of immortalised cell lines

Single cell suspensions were serially replated in methylcellulose medium supplemented with the same growth factors without G418. Cell lines were established by picking single colonies following the third round of plating and propagation in DMEM liquid medium containing 10% FCS, L-glutamine, and 50 μM 2-mercaptoethanol in the presence of TPO, and SCF or TPO, SCF and, IL-3.

2.9 Cytokine withdrawal assay

Cells were seeded at 3×10^3 per well of a 96 well plate. Cells were washed and cultured in DMEM (10% FCS, 2 mM L-glutamine and 50 μM 2-ME) supplemented with various combinations of cytokines (50 ng/mL TPO, 100ng/mL SCF and 10 ng/mL IL-3). Cells were assayed for viability using the CellTiter 96® AQ_{ueous} One Solution (Promega) reagent, added into each well and resuspended thoroughly. The cells were incubated in the dark at 37°C and absorbance was measured at 495 nm using Microplate reader 680 (Biorad).

2.10 Cytospins

To analyse cellular morphology, cells were removed from methylcellulose cultures and washed twice in 10 mL PBS and 0.1% BSA buffer. Cells were resuspended at a concentration of 3×10^5/mL in the wash buffer. Slides were placed on a metal holder and then a cytofunnel (VWR) was placed on top. 100 μl cells were carefully dropped into the funnel. The cells were centrifuged for 5 min at 500 rpm with low deceleration in a Shandon Cytospin 3 (ThermoElectron Corporation).

2.11 Wright-Giemsa staining

The slides were removed and air dried for 10 minutes prior to staining. The slides were stained using a Shandon varistain 24-4 automated staining machine in the Haematology department at Institute of Child Health (ICH).

2.12 INT staining

Colonies were stained with 1 mg/mL *p*-iodonitrotetrazolium (INT) (Sigma) in phosphate-buffered saline (PBS). The colonies were scanned on a GS-800 calibrated densitometer (Biorad) 48 hrs following staining.

2.13 Histopathology

Murine tissues were fixed in 10% neutral buffered formalin and were parafin embedded. Hematoxylin and Eosin (H&E) staining was carried out on 4-μM sections. This was carried out by Dr Sebire in the Department of Pathology at the Institute of Child Health (ICH).

2.14 Determination of viral titre

NIH3T3 cells were seeded the day before at a density of 2.0×10^5 cells per well of a 6 well plate. Viral supernatant was harvested from LinXE cells 48 hrs after transduction and diluted 1:20, 1:200 and 1:2000 in complete DMEM medium. NIH3T3 cells were transduced with 2 mL of each virus dilution in the presence of 5 μg/mL polybrene. Infection efficiency was measured 48 hrs after infection by

measuring hCD2 expression levels. The viral titre was calculated using the following formula:

Viral titre (infectious particles / mL):

$$\frac{\%\text{ of CD2 positive cells} \times \text{dilution factor}}{2} \times (4\times10^5)$$

2.15 NOD/SCID and C57BL/6 Mice

All mice were maintained in the animal facilities of the National Institute for Medical Research, and the Institute of Child Health. Experiments were performed according to Home Office regulations and National Institute for Medical Research (NIMR) and Institute of Child Health (ICH) institutional guidelines.

2.16 *In vivo* transplantation

Retrovirally transduced cells were intravenously injected into sub-lethally irradiated (6 Gγ) recipient C57BL/6-CD45.1 mice 48 hours after infection. Reconstitution was determined 4 weeks later by flow cytometric analysis of peripheral blood. Donor C57BL/6 cells were distinguished from recipient C57BL/6-CD45.1 cells by the presence of CD45.2 and CD45.1 markers, respectively.

For secondary transplants, cells (10^6) isolated directly from primary leukaemic mice were intravenously injected into sub-lethally irradiated (6Gγ) recipient C57BL/6 mice.

2.17 5-Fluorouracil (5-FU) Treatment

C57BL/6 mice were intravenously injected with 150 mg/kg of 5-Fluorouracil (5-FU) (Faulding Pharmaceuticals). Treated mice were sacrificed 4 days post-treatment and BM cells were isolated.

2.18 Flow Cytometry

Cells were stained with fluorochrome conjugated monoclonal antibodies and isotype control antibodies. For the analysis of leukaemic cells, red cells were lysed in ammonium chloride solution at RT for 10 min prior to primary antibody staining. The cells were washed in staining buffer (PBS with 0.05% sodium azide and 1% bovine serum albumin [BSA]) and pre-incubated with unlabeled anti-Fcγ II/III receptor $mAb^{2.4G2}$ for 15 minutes on ice. Cells were stained with monoclonal antibodies conjugated with fluorecein isothiocyanate (FITC), phycoerthyrin (PE), allophycocyanin (APC) or biotin. Biotin conjugated monoclonal antibodies were detected using Peridinin Chlorophyll Protein (PerCP) or PE conjugated strepavidin. Cells were washed twice in the staining buffer and flow cytometry of the cells was carried out on either a Cyan ADP analyser and Summit 4.1 software (Dakocytomation) or a Beckman Coulter Epics XL analyser and EXPO3 software (Beckman Coulter).

For staining of peripheral blood (PB), recipient mice were bled four weeks after transplantation. Blood was washed with 3 mL of FACS staining buffer. 100 µl of primary antibody and anti-Fcγ II/III receptor block was added and incubated for 30 min on ice in dark. Samples were washed in 300 µl of wash buffer. 100 µl of

secondary antibody and anti-Fcγ II/III receptor block was added and incubated on ice for 30 min in dark. To lyse the red blood cells, 2 mL of lysis buffer was added to the samples and incubated at RT for 10 min. The samples were washed with PBS and centrifuged at 370g for 5 min at 4°C. The cells were resuspended in 300 µl of PBS+azide and analysed by Flow cytometry.

Table 5: Flow Cytometry antibodies and working dilutions

Antibody	Clone	Isotype	Supplier	Dilution
Anti-CD45.1 PE	**A20**	**IgG2a κ**	**eBiosciences**	**1:100**
Anti-CD2 PE	LF-A2	IgG1 κ	eBiosciences	1:100
Anti-cKit PE	**2B8**	**IgG2a κ**	**BD Pharmingen**	**1:100**
Anti-B220 PE	RA3-6B2	IgG2a κ	BD Pharmingen	1:100
Anti-IgG1 PE	**A85-1**	**IgG1 κ**	**BD Pharmingen**	**1:100**
Anti-IgG2A PE	G155-178	IgG2a κ	BD Pharmingen	1:100
Anti-IgG2B PE	**MPC-11**	**IgG2a κ**	**BD Pharmingen**	**1:100**
Anti-CD45.2 FITC	104	IgG2a κ	eBiosciences	1:100
Anti-Mac1 FITC	**M1/70**	**IgG2b κ**	**BD Pharmingen**	**1:100**
Anti-CD41 FITC	MW/Reg30	IgG1 κ	abcam	1:100
Anti-cKit APC	**2BB**	**IgG2b κ**	**eBiosciences**	**1:250**
Anti-B220 APC	RA3-6B2	IgG2a κ	eBiosciences	1:250
Anti-Gr-1 APC	**RB6 8CS**	**IgG2b κ**	**eBiosciences**	**1:500**
Anti-CD45.2 Biotin	104	IgG2a κ	eBiosciences	1:100
Anti-CD2 Biotin	**LFA2**	**IgG2bg**	**BD Pharmingen**	**1:100**
Strepavidin PE	N/A	N/A	BD Pharmingen	1:250
Strepavidin Prcp	**N/A**	**N/A**	**BD Pharmingen**	**1:350**

2.19 Southern Blotting

Cells were centrifuged and pellets lysed at 5-10×10^6 and lysed in 400 µl TNES (TE, 0.1 M NaCl, 0.1% SDS) containing 0.5 mg/mL proteinase K (Roche) and incubated O/N at 37°C. For DNA extraction, 0.5 mL of phenol-chloroform-isoamylacohol (25 parts phenol, 24 parts chloroform, 1 part Isoamylalcohol) was added and shaken vigorously for 1 min. The top phase was removed and transferred into a tube containing 1 mL ethanol: 3M NaOAC (25:1) volume ratio and mixed by vortexing. DNA was precipitated by incubating on dry ice for 2 hours. The precipitated DNA was centrifuged at 13000 rpm for 10 min. The supernatant was removed and added to 1 mL of 70% ethanol and centrifuged again for 2 min. The DNA was dissolved in 100 µl TE (pH 8.0). 10 µg of the purified DNA was digested O/N at 37°C with specific restriction enzymes in the presence of 10 mg/mL of BSA and 10 mg/mL of RNase.

The digested DNA was loaded on a 0.8% agarose gel and electrophrosed overnight at 35V. The DNA on the gel was denatured by soaking the gel in a tray of denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 45 min and the solution was changed every 15 min. The gel was washed in H_2O and then soaked in neutralisation solution (1 M Tris (pH 7.4), 1.5 M NaCl) for 30 min with gentle agitation. The gel was further washed with 10× SSC (National Diagnostics) and the DNA was transferred on to a nylon membrane (Amersham) O/N. The DNA was fixed onto the membrane by incubation at 80°C for 2 hrs. To prevent any non-specific binding, the membrane was placed in a pre-hybridisation solution (5× SSC, 0.5% SDS, 0.1 g/mL dextran sulphate (Sigma-Aldrich), 5× Denhardts (Sigma-

Aldrich) and 0.5 mg/mL salmon sperm (Sigma-Aldrich) for 2 hrs at 65°C with gentle rotation.

DNA probe labelling and purification was carried out using the Ready-To-Go™ DNA labelling beads and Probe Quant™ 96 G-50 Micro Columns, according to the manufacture's instructions (GE Healthcare). The blot was hybridised with the probe at 65°C O/N with gentle rotation. The blot was washed twice with several wash buffers (wash buffer A: 2× SSC and 0.1% SDS at 55°C for 30 min, wash buffer B: 0.5× SSC and 0.1% SDS at 55°C for 20 min). The bands were visualised using a typhoon phosphorimager (GE Healthcare).

2.20 Immunobloting

The protein lysates were resuspended in 2× SDS sample buffer, boiled for 5 min and were separated on a polyacrylamide gel. Protein samples were resolved on a 10% SDS-PAGE (sodium dodecylsulfate-polyacrylamide gel electrophoresis). The main lower gel composed of 10% or 15% acrylamide gel (acrylamide:bisacylamide 37.5:1, 1.5 M Tris-HCl (pH 8.5) and 2% SDS). The gel was polymerised by the addition of 10% APS (ammonium per sulphate) and 0.01% TEMED (N,N,N′,N′-Tetramethylethylenediamine). The stacking gel composed of 5% acrylamide gel (acrylamide:bisacylamide 37.5:1, 1.0 M Tris-HCl (pH6.8) and 2% SDS). The samples were run in 1× running buffer (1.92 M glycine, 250 mM tris-base, 1% SDS) overnight at constant voltage of 50 V. The proteins were transferred onto a PVDF (polyvinylidenfluoride) membrane (Immobilon-P, Millipore) using 9.5 mM CAPs (N-Cyclohexyl-3-aminopanesulfonic acid, pH 11.0) for 5 hrs at 100 V. The

membrane was blocked in PBS with 5% nonfat milk and 0.2% Tween-20 for 1 hr at room temperature. The membrane was incubated with specific primary antibodies overnight at 4°C with constant shaking, washed 3× with PBS-Tween and probed with a secondary antibody for 1 hour at room temperature. The membrane was washed 3× with PBS-Tween and proteins were detected using ECL reagent (Amersham) according to the manufacturer's instructions.

Table 6: Immunoblotting antibodies

Antibody	Clone	Supplier	Dilution
Rabbit anti-ERG-3 1/2/3	C-17	Santa-Cruz	1:2000
Mouse anti-FLAG	M2	Sigma-Aldrich	1:2000
Mouse anti-Myc	9B11	Cell signalling technologies	1:1000
Goat anti-actin	I-19	Santa-Cruz	1:1000
Mouse anti-NPM	Fc-61991	Zymed	1:2000
Rat anti-Tubulin	YL1/2	Serotec	1:1000
Mouse anti-GAL4	GAL4-8	Zymed	1:1000
anti-mouse HRP	N/A	Santa-Cruz	1:2000
anti-rat HRP	N/A	Santa-Cruz	1:2000
anti-goat HRP	N/A	Santa-Cruz	1:2000
anti-rabbit HRP	N/A	Santa-Cruz	1:2000

2.21 Immunoprecipitation

Mouse NIH3T3 fibroblasts were lysed in RIPA (radioimmunoprecipitation assay) lysis buffer (50 mM Tris HCL, pH 7.5, 150 mM NaCL, 1 mM EDTA, 1% NP40,

0.5% sodium deoxycholate and 0.1% SDS) containing 1× complete protease inhibitor tablets (Roche Applied Science). Cell lysates were pre-cleared using protein A coupled agarose beads (Roche) for 1hr at 4°C. The pre-cleared supernatant was removed and 10 μg/mL mouse NPM antibody (FC-61991-Zymed) or 3 μg/mL mouse FLAG antibody (M2-Sigma-Aldrich) were used to precipitate complexes. The antibody-protein complex was incubated overnight at 4°C with constant rotation. Appropriate beads were added to the protein samples for 1 hr at 4° C. The immunoprecipitates bound to the beads were collected by centrifugation and washed several times with 1 mL of cold lysis buffer. The beads were boiled for 5 min in 2× SDS sample buffer (125 mM Tris-HCL pH 6.8, 4% SDS, 5% 2-mercaptoethanol, 20% glycerol and 0.1% bromophenol blue). The precipitated proteins were separated on SDS-PAGE under reducing conditions. The mouse anti-FLAG antibody and mouse NPM antibody (Zymed) were used for immunoblotting. Mouse anti-GAL4 antibody (Zymed) was used as an IgG-κ isotype control in immunoprecipitations.

2.22 Luciferase reporter assay

Mouse NIH3T3 fibroblasts were transiently transfected with 0.4 μg of pCDNA3 expression vector, 0.4 μg pCDNA3-*NPM-MLF1* or with 0.4 μg pCDNA3-*NPM*. Cells were cotransfected with 50 ng p53 luciferase construct (el-Deiry *et al.*, 1992) containing 13 repeats of p53 consensus sequence (G/A G/A G/A C A/T T/A G C/T C/T C/T). Cells were also transfected with or without a FLAG tagged-p53 expression vector (0.2 μg) obtained from Dr El-Deiry (el-Deiry *et al.*, 1992). Mutant p53 luciferase construct containing 15 repeats of p53 consensus sequence mutated at

four "hot spots" (el-Deiry *et al.*, 1992) was also used as a negative control. Renilla luciferase reporter construct (50 ng) was used as an internal control to normalise for transfection efficiency. Luciferase activity was measured in triplicate samples 24 hr after transfection with a dual-luciferase reporter system (Promega) according to the manufacturer's instructions. A luminometer LB 9507 machine was used for reporter assay measurement.

2.23 Immunofluorescence

Mouse NIH3T3 cells grown on coverslips were fixed in 4% paraformaldehyde. Cover slips were washed 3× in PBS and permeabilised in 0.5% Triton X-100 for 5 min at room temperature. Cells were washed in PBS and blocked for 30 min with 50% horse serum in PBS. Immunolabelling of the cells was carried out for 1 hour with the anti-FLAG M2 monoclonal antibody (Sigma). Cells were washed in PBS and were subsequently incubated for 1 hour with Rhodamine conjugated anti-IgG antibody (Immunoresearch Laboratories). After several rounds of washing the nuclei was stained with 10 μg/mL Hoechst dye for 5-10 min at room temperature. Following two rounds of washing, coverslips were mounted using Citifluor and nail varnish. Slides were examined using a Nodel Axiplan 2 Fluorescence microscope (CarlZeiss MicroImaging).

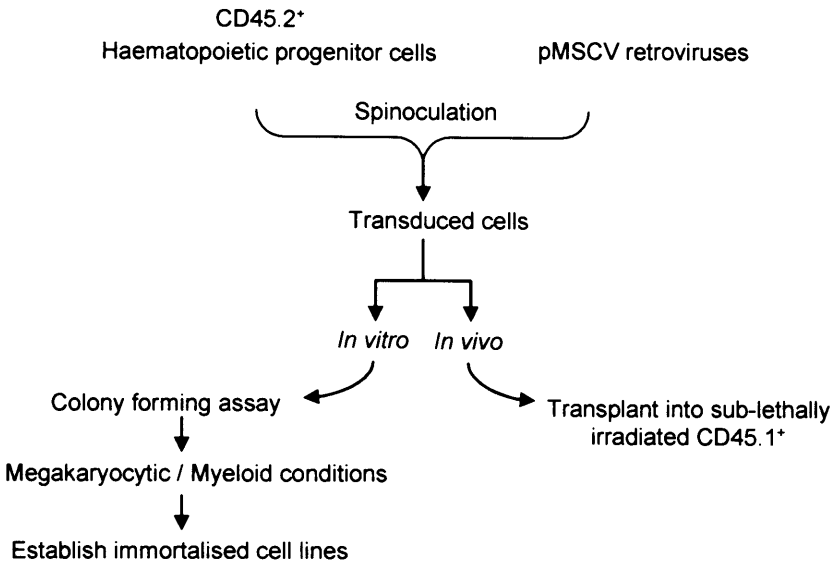


Figure 2.2. Experimental strategy. Embryonic day 12.5 (E12.5) liver was extracted from C57BL6/CD45.2$^+$ mice. Haematopoietic progenitor cells (HPCs) were isolated from foetal liver and cultured in myeloid conditions (SCF, IL-3 and IL-6). HPCs were transduced with retroviral vectors expressing the gene of interest. The effect of the genes on HPC development was addressed *in vitro* by performing colony forming assays and by transplantation assays *in vivo*. For *in vitro* studies, transduced cells were cultured in methylcellulose media in pro-myeloid (GM-CSF) or pro-megakaryocytic (TPO and SCF) conditions. Cells were cultured at 37°C for 5-10 days, after which the colonies formed were counted and single cells were analysed by flow cytometry. Serial re-plating of the transduced cells was carried out following initial selection of neomycin resistant cells in the first round of plating. The immortalisation capacity of the specific genes was investigated by growth of cells from the 3rd round of plating in liquid culture. For *in vivo* studies, transduced cells expressing genes of interest were injected into sub-lethally irradiated mice to determine the potential contribution of these genes to leukaemic development.

Chapter 3

Results

ERG-3 promotes megakaryopoiesis

Results

Chapter 3: ERG-3 promotes megakaryopoiesis

DS children have a 20-fold increased risk of childhood acute lymphoblastic leukaemia (ALL) and a 600-fold increased risk for acute megakaryoblastic leukaemia (AMKL). Around 10% of DS newborns have a transient myeloproliferative disorder (TMD) that resolves spontaneously but approximately 20% of TMD patients will progress to develop AMKL during early childhood. It has been proposed that overexpression of one or more genes on chromosome 21 are crucial in the leukaemogenic transformation of megakaryoblasts. Several candidate genes have been identified such as *RUNX1*, *BACH1*, *ETS2* and *ERG*. ERG is a potentially good candidate as the ERG-3 haematopoietic isoform has been shown to be expressed in DS and non-DS AMKL patient samples and to be involved in megakaryocytic differentiation (Rainis et al., 2005). This study also identified *ERG-3* as the main haematopoietic *ERG* isoform. Truncated forms of ERG are involved in several oncogenic fusion translocations in leukaemia, Ewing sarcoma and prostate cancer. Overexpression of ERG has been recently reported to characterize myeloid leukaemias with a particularly poor outcome, however it is unclear if this overexpression is simply correlative or has a direct oncogenic role in these leukaemias. We set out to investigate the role of ERG-3 in murine megakaryopoiesis.

3.1 TPO and SCF cytokines give optimal conditions for MK development

Our experimental strategy was to transduce foetal HPCs with the appropriate retrovirus and carry out colony forming assay under cytokine conditions that support limited megakaryopoiesis. Different cocktails of cytokines were used to determine the cytokine combination that is required for megakaryocyte differentiation and HPC survival and growth. HPCs isolated from E12.5 FL were cultured in methylcellulose in the presence of TPO (thrombopoietin) alone, TPO and SCF (stem cell factor), TPO and IL-3 (Interleukin-3) or TPO, IL-3 and IL-6 (Interleukin-6). Cells were harvested at 5 days and 10 days following culture and analysed for the expression of CD41, a marker of megakaryocyte differentiation, and c-Kit, a marker expressed on HPCs. At days 5 and 10 the percentage of cells expressing CD41 was higher in the presence of TPO than in the presence of other cytokine combinations (**Figure 3.1A**). In this context, there was megakaryocytic differentiation in the presence of TPO alone but very few colony formation (**Figure 3.2**), further demonstrating that in addition to TPO, there is a requirement for other growth factors that promotes HPC survival and colony formation. The percentage of CD41 expression was reduced in the presence of SCF but was not greatly altered in the presence of IL-3 or IL-6. However, the number of colonies was increased 2-fold with IL-3 and 3-fold with IL-3 and IL-6. Interestingly, IL-3 has been reported to promote megakaryocyte colony formation. We chose the TPO and SCF cytokine combination to carry out *in vitro* megakaryocyte colony forming assays. Since IL-3 also drives megakaryocytic proliferation, this cytokine was omitted from cultures in order to avoid masking of potential proliferative activity of ERG-3.

3.3 Generation of *ERG-3*, *Gata1* and *Gata1s* retroviral constructs

The *Gata1* and *Gata1s* cDNAs were cloned into the murine stem cell (MSCV)-based retroviral vector, pMSCV, containing a neomycin resistance (neo^r) gene. The *ERG-3* cDNA was cloned into the pMSCV-IRES-hCD2 retroviral vector, which expresses the human CD2 (hCD2) gene lacking the cytoplasmic signalling domain (tailles). The internal ribosome entry site (IRES) allows the translation of two proteins from a bicistronic mRNA (**Figure 3.3A**). The expression of GATA1, GATA1s and ERG-3 proteins was confirmed by Western blot analysis of LinXE cells transfected with pMSCV-*Gata1*, pMSCV-*Gata1s* and pMSCV-*ERG-3* retroviral constructs (**Figure 3.3B**). An anti-Flag antibody revealed the presence of the 50 kDa GATA1 protein and the 40 kDa GATA1s protein, whereas the anti-ERG-3 antibody revealed the presence of a 53 kDa ERG-3 protein.

3.4 ERG-3 promotes megakaryopoiesis

A function for ERG-3 in megakaryopoiesis was demonstrated by the ectopic expression of the protein in HPCs isolated from E12.5 Foetal liver. HPCs were cultured in methylcellulose in the presence of TPO and SCF. Colonies were counted after 7-10 days and single cells were analysed by flow cytometry. Cells transduced with vector alone generated few colonies in the first round of plating and these cells could not be replated into the subsequent round. Cells expressing ERG-3 produced around 50 colonies, however the number of colonies formed was markedly reduced in the second round (**Figure 3.4A**) and these cultures failed to re-plate subsequently (data not shown). Analysis of colony morphology revealed that cells transduced with vector formed very small compact colonies and the ERG-3 expressing cells

formed large sparse colonies in the presence of TPO and SCF (**Figure 3.4B**). Wright-Giemsa staining of ERG-3 cultures revealed presence of mature megakaryocytes (**Figure 3.4C**). Analysis of ERG-3 expressing cells harvested from the first round of plating revealed 45% percent of CD41 expression as compared with 21% CD41 expression in cells transduced with vector alone (**Figure 3.5**). These data demonstrate that ERG-3 is involved in megakaryocytic development.

3.5 ERG-3 immortalises progenitor cells in the presence of IL-3

Since ERG-3 did not immortalise progenitors in the presence of TPO and SCF, we attempted to identify a cytokine combination capable of supporting the potential immortalisation capacity of ERG-3 expressing cells in colony forming assays. Cells transduced with ERG-3 were cultured in the presence of TPO, SCF, IL-6 and IL-11. Both IL-6 and IL-11 are added to the cultures as they are associated with enhanced megakaryocyte proliferation and cell expansion (Lazzari *et al.*, 2000). IL-11 can stimulate the proliferation of the human megakaryocytic cell line, CMK (Kobayashi *et al.*, 1993) and it increases the percentage of high ploidy megakaryocytes derived from both murine and human bone marrow cells, suggesting its role in triggering terminal maturation (Burstein *et al.*, 1992). Furthermore, both IL-6 and IL-11 synergise with TPO and SCF to enhance *ex vivo* expansion of megakaryoblasts from human $CD34^+$ bone marrow cells (Williams *et al.*, 1998). IL-11 and IL-6 also synergise with IL-3 to increase proliferation of progenitors by shortening the G_0 phase of the cell cycle in stem cells (Musashi *et al.*, 1991). In the presence of IL-6 and IL-11, cells transduced with vector alone formed the same number of colonies as cells expressing ERG-3 and control cells failed to form colonies following the first round (**Figure 3.6A**). Cells transduced with ERG-3 ceased to re-plate on to the

fourth round (data not shown). At the third round of plating, there was low number of colonies present in the ERG-3 cultures. The colonies detected were not spread out and some were fused. In contrast, when ERG-3 expressing cells were plated in TPO, SCF and IL-3 alone, they could be serially replated up to the seventh round of plating. IL-3 has megakaryocytic colony stimulating activity and it synergises with TPO to stimulate megakaryocyte production and colony-forming units-megakaryocyte (CFU-MK) (Dolzhanskiy *et al.*, 1997). Cells transduced with vector retrovirus also formed colonies up to the sixth round of plating (**Figure 3.7A**). However, there was a profound difference in the morphology of the colonies formed. ERG-3 transduced cells formed large, hyperproliferative colonies whereas vector transduced cells formed small, intact colonies at each round of plating (**Figure 3.7B**). Furthermore, cells expressing ERG-3 were mostly c-Kit$^+$CD41$^+$ as compared with cells transduced with empty vector which expressed the early progenitor cell marker c-Kit but not CD41 (**Figure 3.7C**).

3.6 ERG-3 immortalised cell lines have an immature megakaryoblastic phenotype

Single colonies were picked from the third round of plating and cultured in liquid in DMEM complete medium supplemented with TPO, SCF and IL-3. Four ERG-3 immortalised cell lines were generated and these had the same rate of growth **(Figure 3.8A)**. Cells transduced with vector alone were not viable in liquid culture (data not shown). The presence of the 53 kDa ERG-3 protein in these cell lines was detected using an anti-ERG-3 antibody **(Figure 3.8B)**. The cell lines were all positive for hCD2 and expressed high levels of c-Kit and low CD41 (**Figure 3.9A**). These cells also had an immature megakaryoblastic phenotype, as shown by Wright-

Giemsa staining (**Figure 3.9B**).

3.7 ERG-3 immortalised cell lines are dependent on SCF and IL-3 for survival

To determine the cytokine dependence of the immortalised cells, an MTS cell viability assay was carried out. Cells were grown in DMEM complete medium supplemented with various combinations of TPO, SCF and IL-3. Cells were also cultured in the absence of IL-3, TPO or SCF. Cell viability was measured 48 hrs and 72 hrs after cytokine withdrawal. At 48 hrs, the absence of TPO did not have any effect on the cell viability whereas there was a significant reduction in cellular growth in the absence of SCF for all four ERG-3 immortalised cell lines. There was an additional reduction in viability in the absence of IL-3 with cell lines E3-1, E3-2 and E3-3 but not E3-4. There was no difference in cell viability between E3-4 supplemented with TPO, SCF and IL-3 and cultures lacking IL-3. In addition, this cell line appeared to be more dependent on SCF than the other three cell lines (**Figure 3.10A**). At 72 hrs, there was a further decrease in viability in comparison with cultures containing SCF and IL-3 with cell lines E3-1, E3-2 and E3-3. In contrast, although there is a reduction in viable cells in the absence of IL-3 as compared with cultures containing TPO, SCF and IL-3 or TPO and IL-3, E3-4 cells appeared to be independent of IL-3 (**Figure 3.10B**).

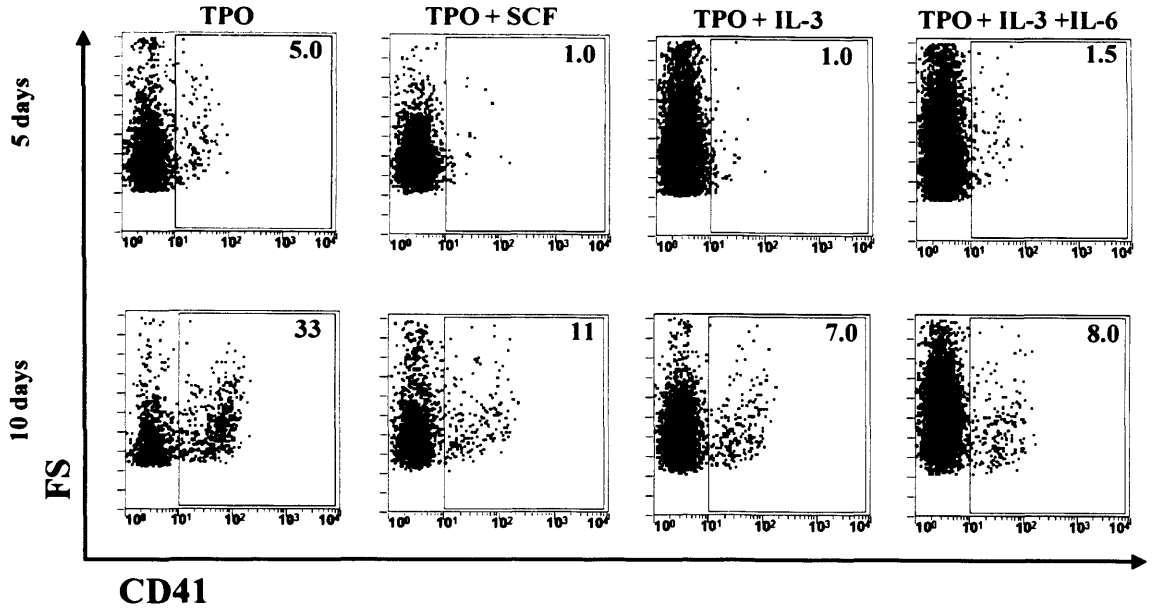


Figure 3.1. TPO and SCF are optimal for megakaryocytic differentiation. Dot plots represent the expression of CD41. The numbers show the percentage of positive cells. HPCs were isolated from E12.5 Foetal liver and were plated in methycellulose medium 3234 in the presence of various cytokine combinations: TPO (50 ng/mL) alone, TPO and SCF (50 ng/mL each), TPO and IL-3 (10 ng/mL) or TPO, IL-3 and of IL-6 (10 ng/mL). Cells were harvested at days 5 and 10. The data is a representative of two independent experiments.

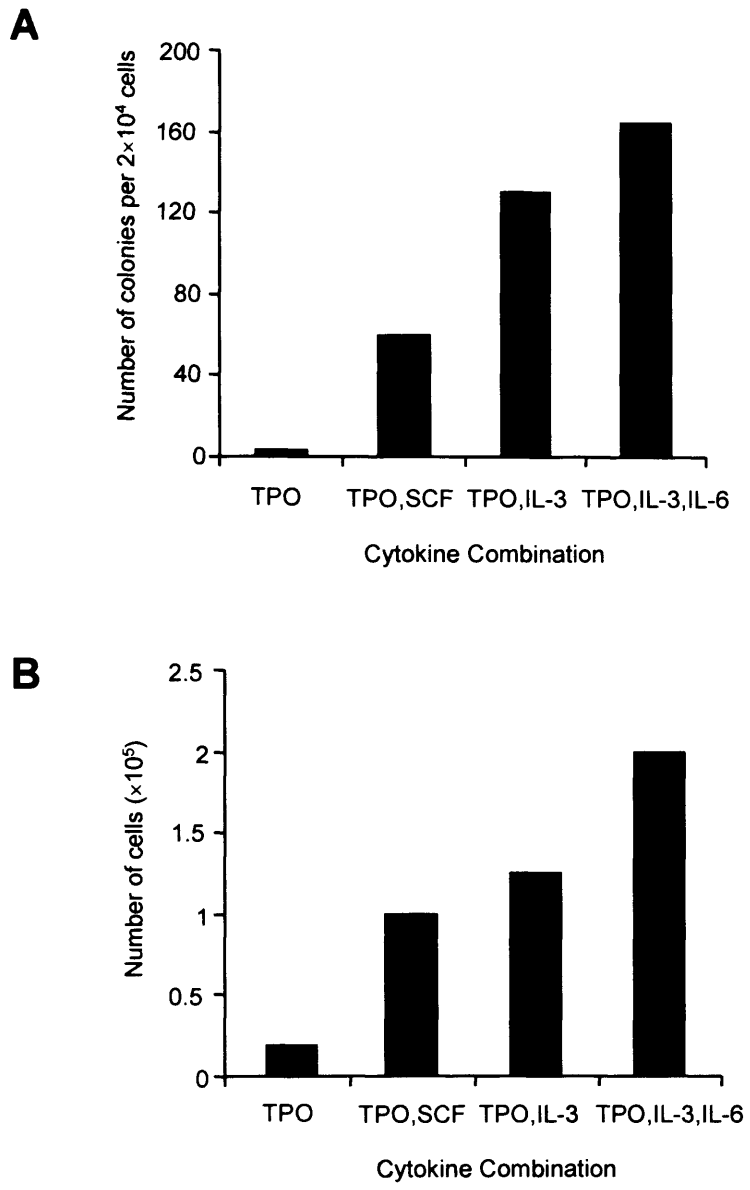


Figure 3.2. TPO and SCF are sufficient for megakaryocytic colony formation. HPCs were isolated from E12.5 Foetal liver and were cultured in methylcellulose media 3234 supplemented with either TPO (50 ng/mL) alone, TPO and SCF (50 ng/mL), TPO, IL-3 (10 ng/mL) or TPO, IL-3 and IL-6 (10 ng/mL). Cells were cultured for 10 days. **A.** Number of colonies produced in the first round of plating. **B.** Total number of cells harvested from the pooled duplicate cultures in the first round of plating.

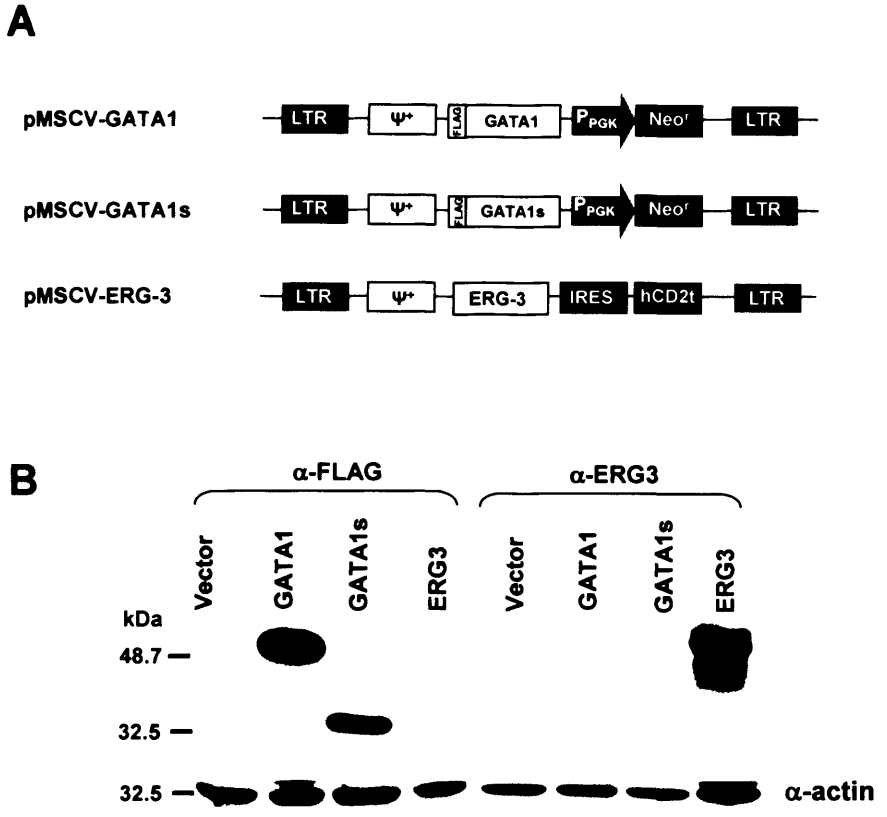


Figure 3.3. Expression of GATA1, GATA1s and ERG-3 in LinXE cells. A. Diagram depicting the retroviral constructs used to express ERG-3, Gata1 (FLAG), and Gata1s (FLAG). **B.** Western blot analysis of protein lysates from LinXE cells transfected with pMSCV-neo, pMSCV-*GATA1*, pMSCV-*GATA1s* and pMSCV-*ERG-3* constructs. FLAG tagged GATA1 and GATA1s proteins were detected using an anti-FLAG antibody and ERG-3 protein was detected using an anti-ERG-3 antibody.

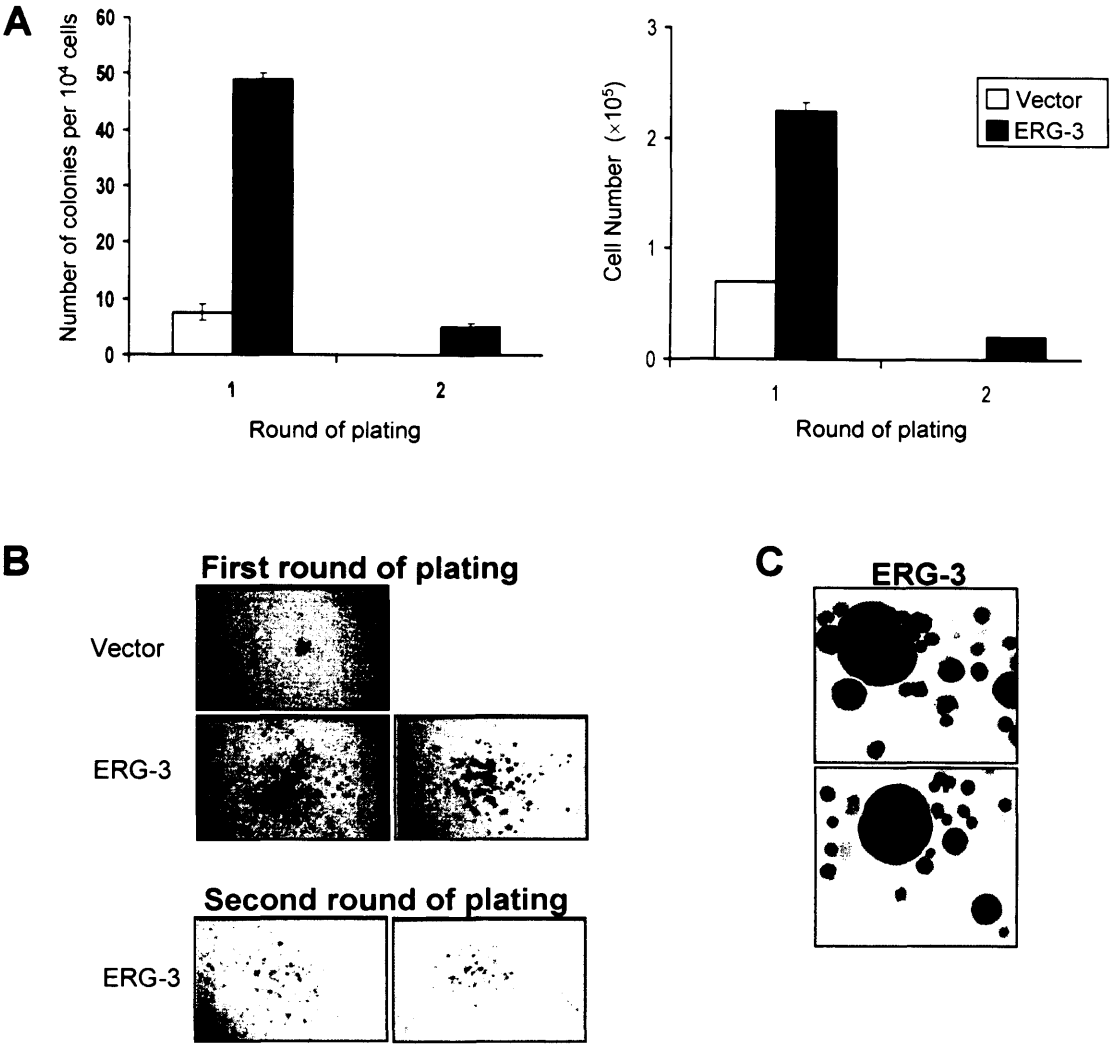


Figure 3.4. ERG-3 promotes megakaryopoiesis. A. Bar chart showing the number of colonies (left panel) and number of cells (right panel) at each round of plating. The data represents mean and ± S.D. of duplicate cultures. HPCs were transduced with vector or *ERG-3* retrovirus and cultured in methylcellulose medium in the presence of TPO and SCF. Colonies were scored and single cells counted. **B.** Typical morphology of colonies formed at the first and second round of plating (original magnification ×40). **C.** Representative Wright-Giemsa staining of cells expressing ERG-3 (original magnification, ×400).

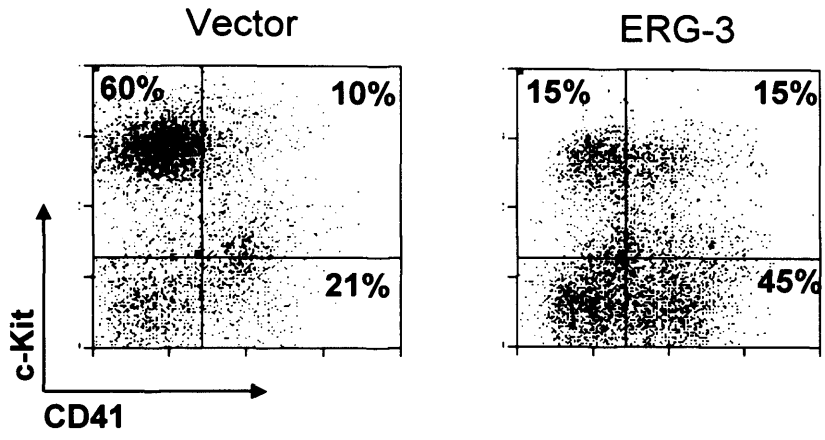


Figure 3.5. Flow cytometry: ERG-3 promotes megakaryopoiesis. HPCs were transduced with vector or *ERG-3* retrovirus and cultured in methylcellulose in the presence of TPO and SCF. Flow cytometry carried out on single cells isolated from the first round of plating. Dot plots show the expression of c-Kit$^+$CD41$^+$ cells within the CD2 positive population.

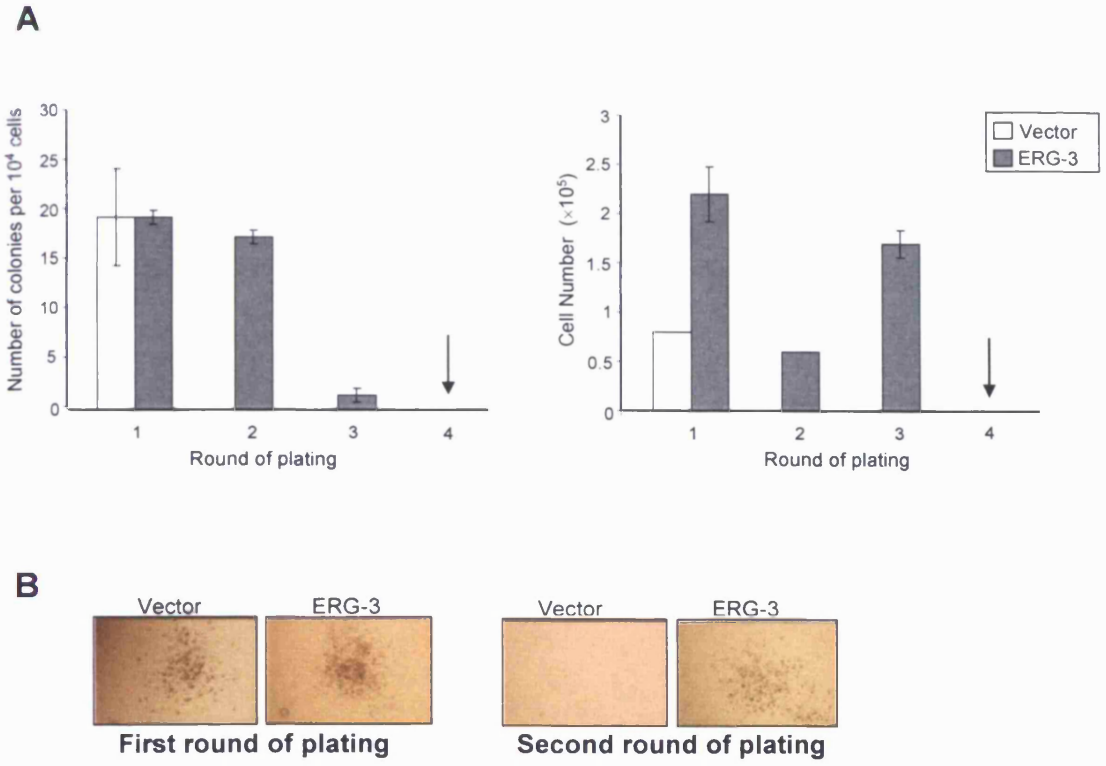


Figure 3.6. ERG-3 does not immortalise HPCs in the presence of TPO, SCF, IL-6 and IL-11. A. Bar chart showing the number of colonies (left panel) and number of cells (right panel) at each round of plating. The data represents mean and ± S.D. of duplicate cultures. HPCs were transduced with vector or *ERG-3* retrovirus and cultured in methylcellulose in the presence of TPO, SCF, IL-6 and IL-11. Colonies were scored and single cells counted. **B.** Typical morphology of colonies formed at the first and second round of plating (original magnification, ×40). The arrows indicate the absence of colony formation and cell number.

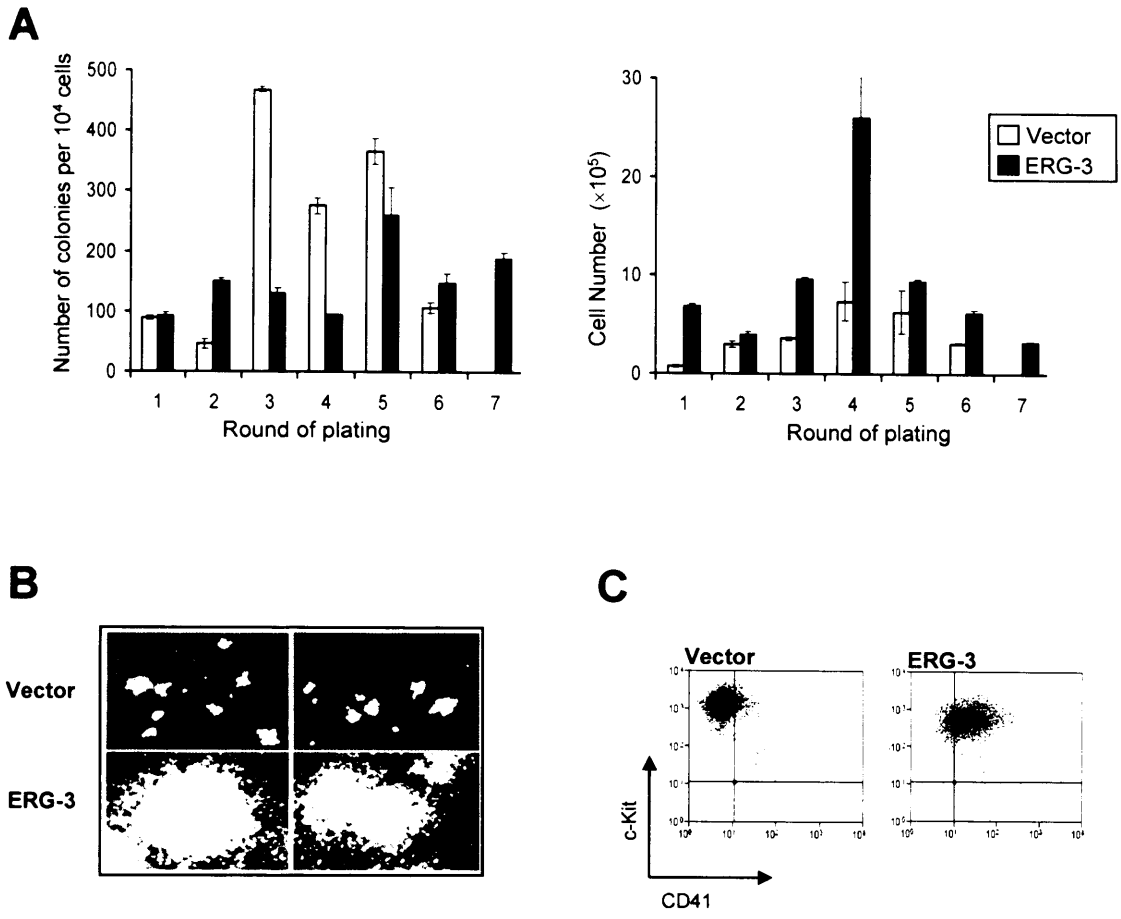


Figure 3.7. ERG-3 immortalises HPCS in the presence of IL-3. A. Bar chart representing the number of colonies (left panel) and number of cells (right panel) at each round of plating. The data represents the mean and ± S.D. of duplicate cultures. HPCs were transduced with vector or with *ERG-3* retrovirus . Colony forming assays were performed by culturing cells in the presence of TPO (50ng/ml), SCF (100ng/ml) and IL-3 (10ng/ml) for 6-10 days. Colonies were scored and the total cell number was counted from third round of plating. **B.** Representative morphology of colonies formed by transduced cells (original magnification, ×100). **C.** Dot plots represent c-kit$^+$CD41$^+$ expression in cells transduced with Vector or ERG-3.

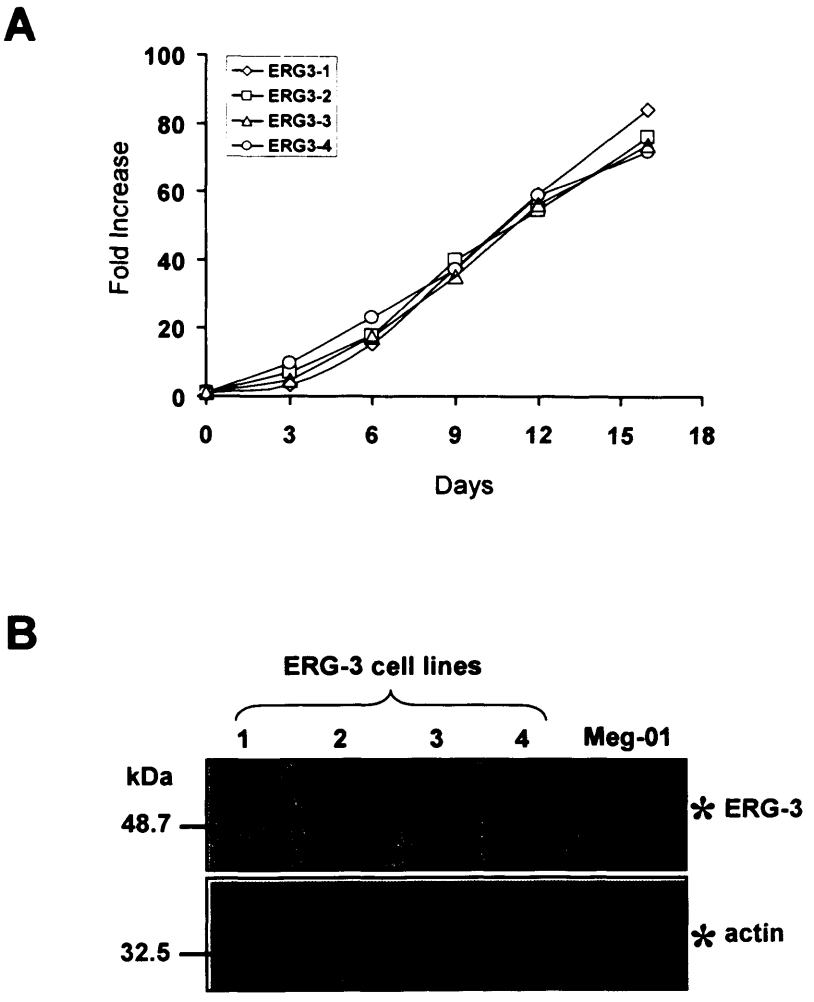


Figure 3.8. Generation of ERG-3 immortalised cell lines. Single colonies were picked and cultured in the presence of TPO, SCF and IL-3. Cells transduced with vector did not survive in liquid culture. **A.** Fold increase in cell number of four ERG-3 immortalised cells lines. **B.** Western blot analysis of protein lysates from ERG-3 immortalised cell lines. The presence of ERG-3 protein was detected using an anti-ERG-3 antibody. Protein lysate from the megakaryoblastic cell line (Meg-01) was used as a positive control for the expression of ERG-3. An anti-actin antibody was used as loading control.

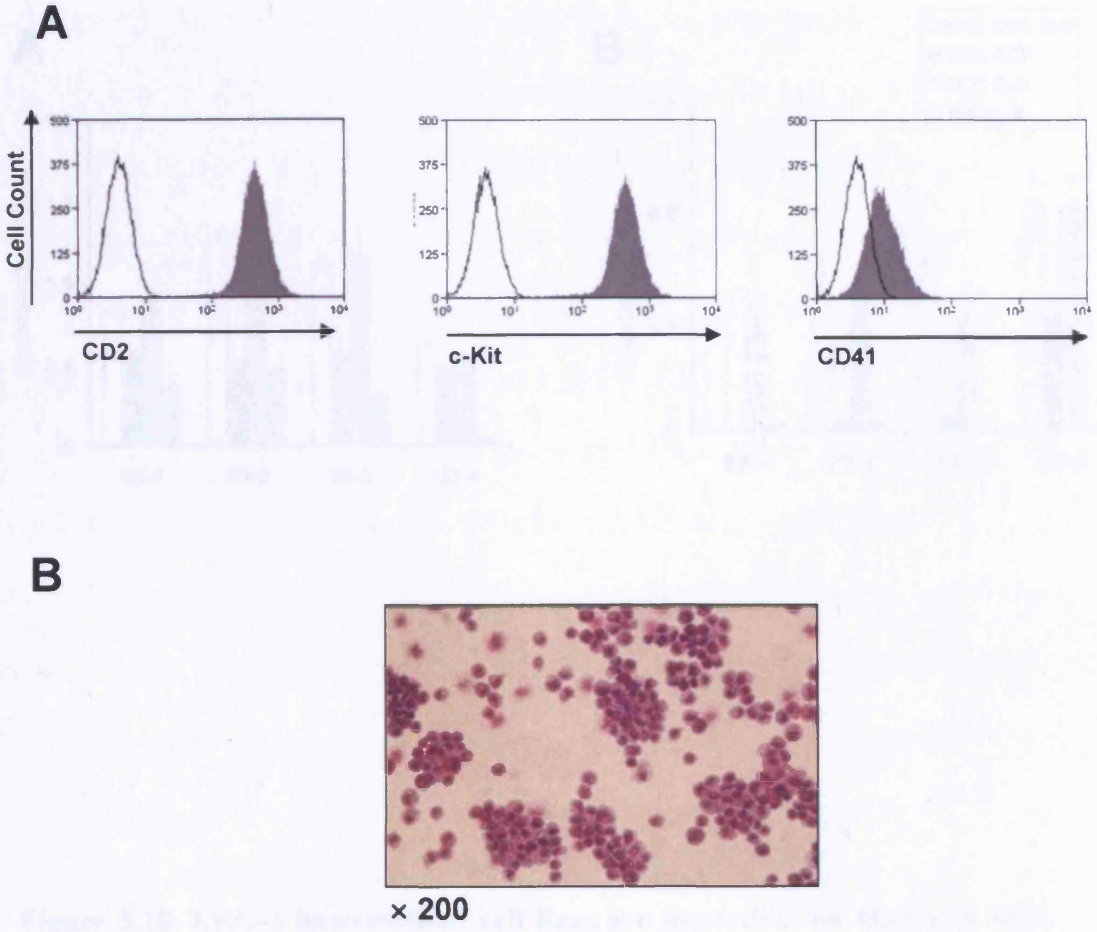


Figure 3.9. Immortalised cell lines have immature megakaryoblastic phenotype.. **A.** Phenotype of a representative ERG-3 immortalised cell line. Histogram depicting the expression of hCD2, c-Kit and CD41. **B.** Representative morphology of ERG-3 immortalised cell line as demonstrated by Wright-Giemsa staining, (Original magnification × 200).

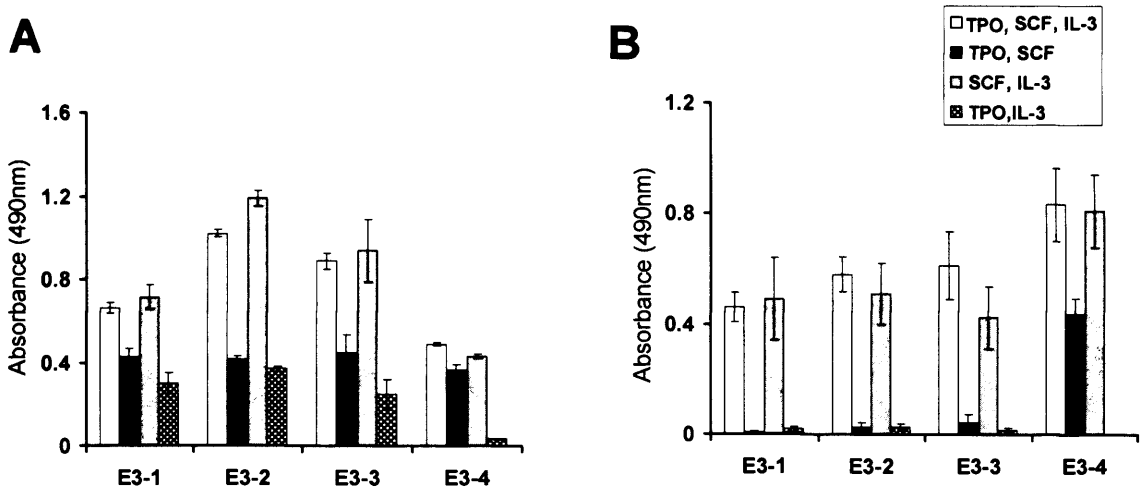


Figure 3.10. ERG-3 immortalised cell lines are dependent on IL-3 and SCF. The cytokine requirement of each ERG-3 immortalised cell line was assessed using an MTS assay. Each cell line was grown in the presence of TPO, SCF and IL-3, TPO and SCF, SCF and IL-3 or TPO and IL-3. MTS assay was carried out 48 hrs (**A**) or 72 hrs (**B**) following cytokine withdrawal. Absorbance was measured at 490 nm. The data represents mean and ± S.D. of duplicate cultures.

3.8 Discussion

The molecular mechanism for leukaemogenesis in DS is as yet unclear. Particular genes on chromosome 21 have been implicated in the pathogenesis of DS but not much is known about what causes abnormal megakaryopoiesis in these patients. There are a number of genes located on chromosome 21 that are involved in the regulation of different aspects of haematopoiesis. These genes, in combination with *GATA1* mutation may be contributing factors in leukaemic evolution in DS. *RUNX1*, *BACH1* and *ETS* related genes (*ETS1*, *ETS2* and *ERG*) are few of the candidate genes, located in the Down syndrome critical region (DSCR), that have been shown to have a role in megakaryocytic development. RUNX1 is a transcription factor which plays a vital role in gene regulation in the haematopoietic system. The importance of RUNX1 as the positive regulator of megakaryopoiesis was revealed in a conditional knock out mouse in which *Runx1* was deleted in most of haematopoietic progenitor cells. Megakaryocytic maturation is defective in these mice as measured by the presence of abnormally small immature megakaryocytes, low levels of platelets and aberrant polyploidisation in comparison with wild type mice (Ichikawa *et al.*, 2004). In contrast to RUNX1, BACH1 negatively regulates genes required for megakaryocyte maturation, as shown by the reduction of platelets in the *Bach1* transgenic mice (Toki *et al.*, 2005).

ERG-3 is another gene located on the DSCR on chromosome 21 and data from Rainis *et al* show *ERG-3* mRNA expression in cell lines established from DS patients. In addition, the possible role of ERG-3 in megakaryopoiesis was suggested using *in vitro* studies where ectopic expression of ERG-3 in K562 cells induced

megakaryocyte maturation at the expense of erythropoietic differentiation (Rainis et al., 2005). The aim of our study was to establish if there is any co-operativity between the expression of the *GATA1* mutation, detected in DS patients, and ERG-3. To examine this, we first wanted to determine if ERG-3 had any role in megakaryopoiesis. We generated MSCV-based retroviral vectors to express GATA1, GATA1s or ERG-3 and confirmed their ability to produce each protein by immunoblotting. We used progenitor cells isolated from E12.5 FL, as data from *Gata1s* knockin mice have shown that E12.5 FL contains a significant fraction of hyperproliferative megakaryocytic precursors which diminishes very rapidly at later days in embryonic development.

Colony forming assays were carried out using isolated HPCs in the presence of various cytokine combinations to determine the optimal combination required for megakaryopoiesis. Several cytokine conditions have been used by various groups in colony forming assays. The main haematopoietic cytokine required for commitment of HPCs along the megakaryocytic lineage is TPO. Recombinant TPO stimulates the growth and differentiation of murine megakaryocytes *in vitro* (Wendling *et al.*, 1994). We have shown that although TPO alone induced a marked increase in megakaryocytic maturation, it did not support the growth of megakaryocytic colonies. Indeed, in a study by Guerriero *et al*, megakaryocytes were generated in the presence of TPO alone, but their proliferation was significantly compromised (Guerriero *et al.*, 1995). In addition, TPO has been shown to synergise with SCF, erythropoietin (EPO), IL-3, IL-6 and IL-11 to promote megakaryocytic proliferation, differentiation and platelet production both in human and murine HPCs. In our study, TPO in combination with SCF, IL-3 and IL-6 promoted

megakaryopoiesis. Expansion of early progenitors was observed with the addition of just IL-3 and IL-6. This observation is supported by studies that show that IL-3 promotes the early stages of megakaryocytic development, while inhibiting terminal differentiation (Dolzhanskiy *et al.*, 1998; Murphy and Leavitt, 1999).

ERG-3 was shown to promote megakaryopoiesis in the presence of TPO and SCF. The cells generally exhibited a megakaryoblastic phenotype, as shown by their expression of CD41. However, HPCs expressing ERG-3 were not able to from colonies past the second round or past the third round in some experiments. To determine whether ERG-3 could induce immortalisation of megakaryocytic progenitor cells, the cytokines used in these cultures was altered. It was shown that cells expressing ERG-3 were immortalised in the presence of IL-3 but not in cultures with IL-6 and IL-11. Indeed, IL-6 and IL-11 cooperate with TPO to induce late maturation of megakaryocytes (Koike *et al.*, 1990; Burstein et al., 1992) and the clonogenic potential of progenitor cells in the presence of IL-6 and IL-11 is lower than in cultures with IL-3 alone (Lazzari et al., 2000). Although IL-3 has been shown to inhibit megakaryocytic differentiation (Dolzhanskiy et al., 1997), the presence of ERG-3 promotes commitment and maturation of cells along this lineage. However, control cells that were transduced with vector alone did not express the megakaryocytic marker CD41. Furthermore, all the cells in these cultures were c-Kit positive in the presence of IL-3, which is in contrast to the very low c-Kit expression levels detected in cultures with TPO and SCF alone. This data is in line with current evidence that IL-3 is required for maintenance of early megakaryocytic progenitors (Broudy *et al.*, 1995; Williams et al., 1998). Our study shows that ERG-3 commits progenitor cells to the megakaryocytic lineage and TPO

and SCF synergise with IL-3 to provide an optimal conditions to trigger expansion of HPCs expressing ERG-3.

The mechanism for ERG-3 mediated immortalisation is unknown. For future studies, *ERG-3* deletion constructs can be generated to determine which domains of this transcription factor are essential for megakaryocytic commitment and progenitor immortalisation. The generation of miRNA/siRNA against *ERG-3* would determine whether the immortalised cells were dependent upon the presence of ERG-3. It may be that ERG-3 is essential for the initiation of immortalisation but its presence is not required for survival of immortalised cells. Finally, the generation of cell lines with inducible/conditional expression of ERG-3 would allow the identification of primary ERG-3 target genes, which could ultimately lead to a better understanding of the function of ERG-3 and its potential role in development of DS associated AMKL.

Chapter 4

Results

ERG-3 collaborates with GATA1s to immortalise progenitors *in vitro* and induce leukaemia *in vivo*

Chapter 4: ERG-3 collaborates with GATA1s

GATA1 normally suppresses the proliferation of megakaryocytic and erythroid precursors while promoting their differentiation. Somatic mutations acquired during foetal haematopoiesis in the GATA1 transcription factor, are detected in megakaryoblasts from all DS patients with either TMD or AMKL. Normal haematopoetic cells express both full length GATA1 and GATA1s proteins. The *GATA1* mutations introduce a premature stop codon or interfere with the alternative splicing of exon 2. This leads to initiation of translation from exon 3, replacing full length GATA1 protein with a shorter isoform, GATA1s. However, *GATA1* mutations alone are insufficient for leukaemogenesis, since replacement of GATA1 with GATA1s causes transient proliferation of immature foetal megakaryocytic progenitors but no postnatal haematopoietic abnormalities. In addition, individuals with germline *GATA1s* show no reported malignancies, strongly suggesting that trisomy 21 is essential for leukaemic development.

4.1 ERG-3 cooperates with GATA1*s* to initiate immortalisation of HPCs

Serial methylcellulose replating assays were carried out to investigate the possible cooperative effects between ERG-*3* and GATA1s in transforming murine HPCs. HPCs isolated from E12.5 foetal liver were transduced with retroviruses encoding *ERG-3*, *Gata1*, *Gata1s* alone or *ERG-3* with *Gata1* or *Gata1s* and cultured in megakaryocyte conditions (SCF and TPO). Cells expressing ERG-3 + GATA1 or with ERG-3 + GATA1s, formed more colonies than cells transformed with vector alone or ERG-3, GATA1 and GATA1*s* alone (**Figure 4.1**). This suggests a potential cooperation of ERG-3 with either GATA1 or GATA1s. However, significant

differences were observed between the transduced cells at the third round of plating. Progenitors expressing ERG-3, GATA1 or GATA1s alone exhausted their self-renewal potential and cells expressing ERG-3 + GATA1 failed to re-plate after the third round, indicating that these cells were not transformed. In contrast progenitors expressing ERG-3 and GATA1s continued to re-plate up to fifth round, and in some experiments up to the seventh round of plating (**Figure 4.1**). The increased number of colonies formed by cells transduced with ERG-3 and GATA1s was evident by the staining of cultures with *p*-iodonitrotetrazolium (INT) at each round of plating (**Figure 4.2A**). The colonies formed had a hyperproliferative morphology (**Figure 4.2B**). Cell lines co-expressing ERG-3 and GATA1s were generated from these cultures. These cells were maintained in liquid culture for up to 8 weeks (**Figure 4.3 and 4.4A**). The cytokine dependence of two cell lines was examined using the MTS assay and it was demonstrated that both cell lines were TPO dependent (**Figure 4.4B**). The cell lines generated expressed high levels of c-Kit and CD41 cell surface markers, characteristic of megakaryocyte progenitors (**Figure 4.4C**). Our data shows that ERG-3 co-operates with the GATA1s mutation to immortalize foetal megakaryocytic progenitors. These immortalised cells have the phenotypic characteristics of the megakaryoblasts found in AMKL patients.

4.2 ERG-3 + GATA1s cell lines do not induce leukaemia in NOD/SCID mice

The leukaemogenic potential of the immortalised cell lines were examined by transplanting cells into NOD/SCID mice. The immortalised cell lines expressed the CD45.2 cell surface marker whereas the recipient cells in the NOD/SCID mice were CD45.1 positive. This allowed us to distinguish donor from recipient cells. The analysis of bone marrow, spleen and thymus of injected mice showed that all the

cells were CD45.2 negative and most were CD45.1 positive (**Figure 4.5A**) suggesting that the immortalised cells did not transplant / survive in the NOD/SCID mice after seven months. The CD45.1 negative cells in the thymus and spleen were also negative for CD45.2 and CD2 (data not shown). The analysis of B cell and myeloid cell populations in bone marrow and spleen showed a normal distribution pattern of B220 positive and Mac1 positive cells in these lymphoid organs (**Figure 4.5B**). In contrast, the CD4 positive and CD8 positive T cell population appeared abnormal in these mice (**Figure 4.5B**). The total cell number in the bone marrow and spleen of 8 mice injected with ERG-3 + GATA1s immortalised cells lines were within the normal range (**Figure 4.5C**). These data show that ERG-3 + GATA1s cell lines were not leukaemogenic in the NOD/SCID transplantation model.

4.3 Induction of leukaemia in mice transplanted with ERG-3 or ERG-3 + GATA1s transduced cells.

To test the leukaemogenic potential of ERG-3 and GATA1s *in vivo*, HPCs co-transduced with ERG-3 and GATA1s were directly transplanted into sub-lethally irradiated C57BL/6 mice 24 hours after infection. For controls, cells transduced with vector, *Gata1s* and *ERG-3* retrovirus alone were also transplanted into recipient mice. The infection efficiency was measured by analysing the expression of human CD2 (hCD2). More than 90% of haematopoietic cells were infected with the hCD2 expressing retrovirus (**Figure 4.6A**). Consistent with the published oncogenic function of ERG-3, all recipient mice transplanted with ERG-3 transduced cells succumbed to leukaemia within 100 days. In addition, cells transduced with ERG-3 and GATA1s induced leukaemia with similar latency (**Figure 4.7**). These mice had splenomagly (**Figure 4.8A**) and there was a

significant increase in cell number in the spleen and reduction in cell number in the bone marrow of the leukaemic mice as compared with control (**Figure 4.8B**). This reduced cell number was due to the presence of leukaemic blasts within the bone marrow. Wright-Giemsa staining of the cells isolated from the spleen and the bone marrow revealed the presence of blasts (**Figure 4.9**). Haematoxylin and Eosin (H&E) staining of tissues revealed a significant infiltration of leukaemic cells into the liver but not kidney of mice that underwent transplantation with cells expressing ERG-3 and cells expressing both ERG-3 and GATA1s (**Figure 4.10**). The B lymphocyte follicles and red pulp, containing predominantly erythrocytes, were apparent in the spleens of control mouse. The architectural structure of the spleen was completely masked by the high presence of blasts in the spleen of leukaemic mice and the bone marrow was also filled with leukaemic cells. The presence of ERG-3 and GATA1s protein in the splenocytes of these mice was confirmed by Western blot analysis (**Figure 4.11**). Integration of the *ERG-3* and *Gata1s* provirus in the leukaemic cells was confirmed by Southern blot analysis. The leukaemias were oligoclonal as shown by multiple proviral integrations (**Figure 4.12**). There is a unique, neo integration in mouse #2, as shown by the presence of a single band and two bands detected with the hCD2 probe, shows several *ERG* provirus integrations in the same mouse. The third band, which has a lower intensity is probably due to a partial digest of genomic DNA.

4.4 ERG-3 + GATA1s transduced cells induce a heterogeneous leukaemia

To characterise the phenotype of the leukaemic cells, flow cytometry analysis was carried out on splenocytes of the leukaemic mice. A cohort of 8 ERG-3 + GATA1s and 15 ERG-3 leukaemic mice were examined. As the *ERG-3* cDNA was cloned in

the pMSCV-IRES-hCD2 vector, the expression of hCD2 in the splenocytes was indicative of ERG-3 expression. The leukaemia detected with ERG-3 alone or with ERG-3 + GATA1s consisted of heterogeneous populations of cells with some mice exhibiting cells that are predominantly c-Kit positive whereas others have cells that are mostly CD41 positive (**Figure 4.13**). There was a significant reduction in the percentage of CD41 positive cells detected in mice with ERG-3 + GATA1s as compared with mice with ERG-3 transduced cells but there was no significant difference in the percentage of c-Kit positive cells (**Figure 4.14**). Whilst the leukaemic cells expressed high levels of CD2, they lacked CD45.2 expression. This is consistent with the report suggesting that mature cells of the MegE lineage lack CD45 expression (Forsberg et al., 2006). These data show that ERG-3 alone is oncogenic and it collaborates with GATA1s *in vivo* to alter the proliferation and/or maturation of leukaemic cells.

The transplantability of leukaemias induced by ERG-3 and ERG-3 + GATA1s was examined by tail vein injection of leukaemic splenocytes and bone marrow cells into secondary sub-lethally irradiated recipient mice. The mice injected with the leukaemic cells did not develop leukaemia and remained healthy (data not shown).

4.5 Leukaemic cell lines could not be grown *in vitro*

In order to generate leukaemic cell lines *in vitro*, ERG-3 expressing cells isolated from bone marrow were cultured in methylcellulose under either myeloid or megakaryocytic conditions. In myeloid cultures, leukaemic cells expressing ERG-3 could not be re-plated past the third round of plating. Whilst there was an increase in colony number at the second round, the total cell number was not increased as the

colonies formed were very small (**Figure 4.15**). In megakaryocytic conditions, ERG-3 leukaemic cells failed to form any colonies in the second round (**Figure 4.16**). Leukaemic cells expressing both ERG-3 + GATA1s had limited self renewal potential in myeloid (**Figure 4.17A**) and megakaryocytic conditions (**Figure 4.18A**). In the first round of plating, colonies formed under the myeloid conditions were small and very dense, resembling the morphology of typical immortalised colony. However, the colonies became sparse in the second round after which cells failed to replate (**Figure 4.18B**). In megakaryocytic conditions, the colonies formed were very sparse (**Figure 4.18B**). These data indicate that the conditions used here were not optimal for culturing leukaemic cells expressing ERG-3 or ERG-3 + GATA1s *in vitro*.

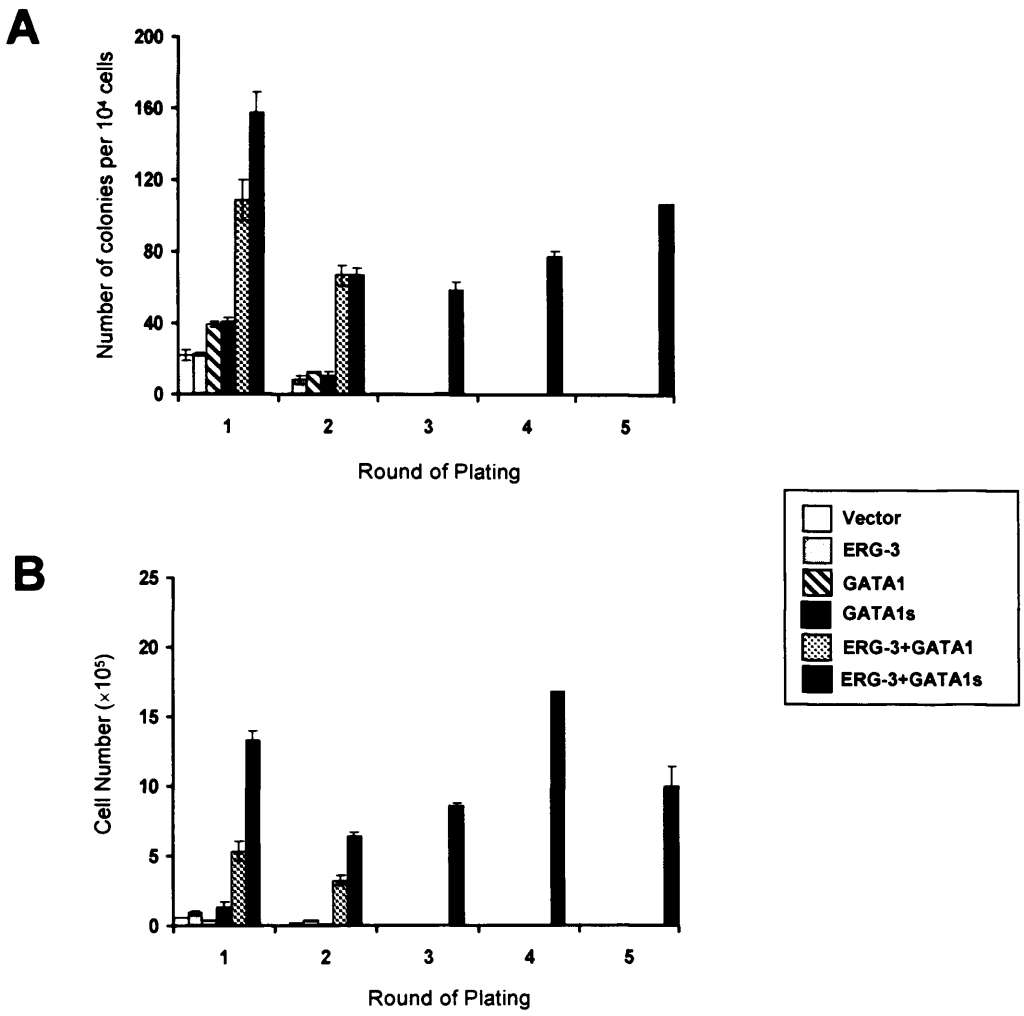


Figure 4.1. ERG-3 collaborates with GATA1s to immortalise HPCs in the presence of TPO and SCF. Bar chart representing the number of colonies (**A**) and number of cells (**B**) at each round of re-plating. The data represents mean and ± S.D. of duplicate cultures. HPCs isolated from foetal liver of E12.5 embryos were transduced with either vector, ERG-3, GATA1, GATA1s, ERG-3 + GATA1 or ERG-3 + GATA1s. Transduced cells were cultured in methylcellulose supplemented with TPO and SCF. Selection (G418) was applied in the first round of plating only.

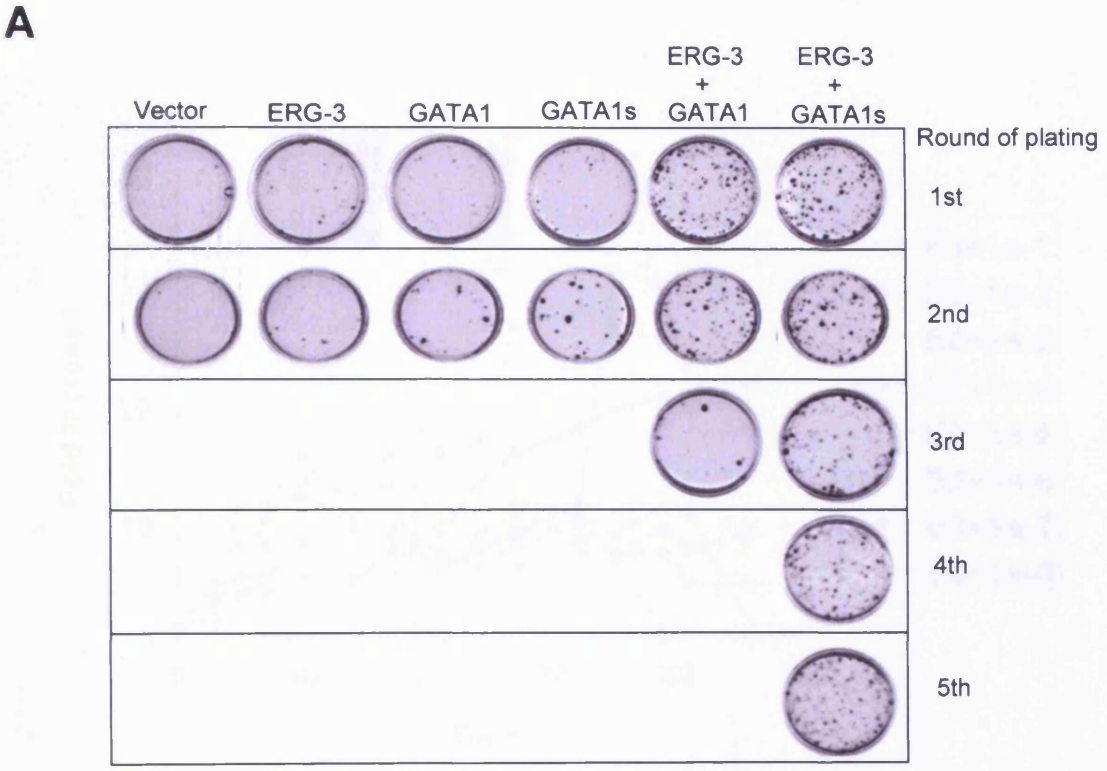


Figure 4.2. ERG-3 + GATA1s immortalise HPCs. A. *P*-iodonitrotetrazolium (INT) stained methylcellulose cultures at each round of plating. **B.** Typical morphology of colonies formed by cells expressing both ERG-3 and GATA1s (original magnification, ×40).

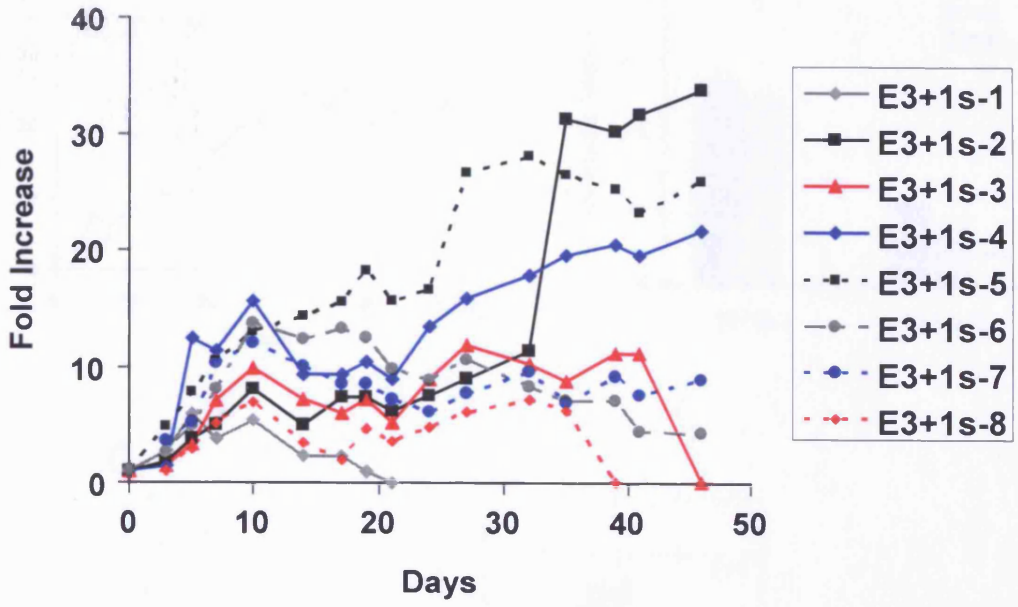


Figure 4.3. Generation of ERG-3 + GATA1s immortalised cell lines. Fold increase in cell number of 8 ERG-3 + GATA1s (E3+1s) immortalised cell lines. Single colonies were picked at the third round of methylcellulose plating and cultured in complete DMEM medium supplemented with TPO and SCF.

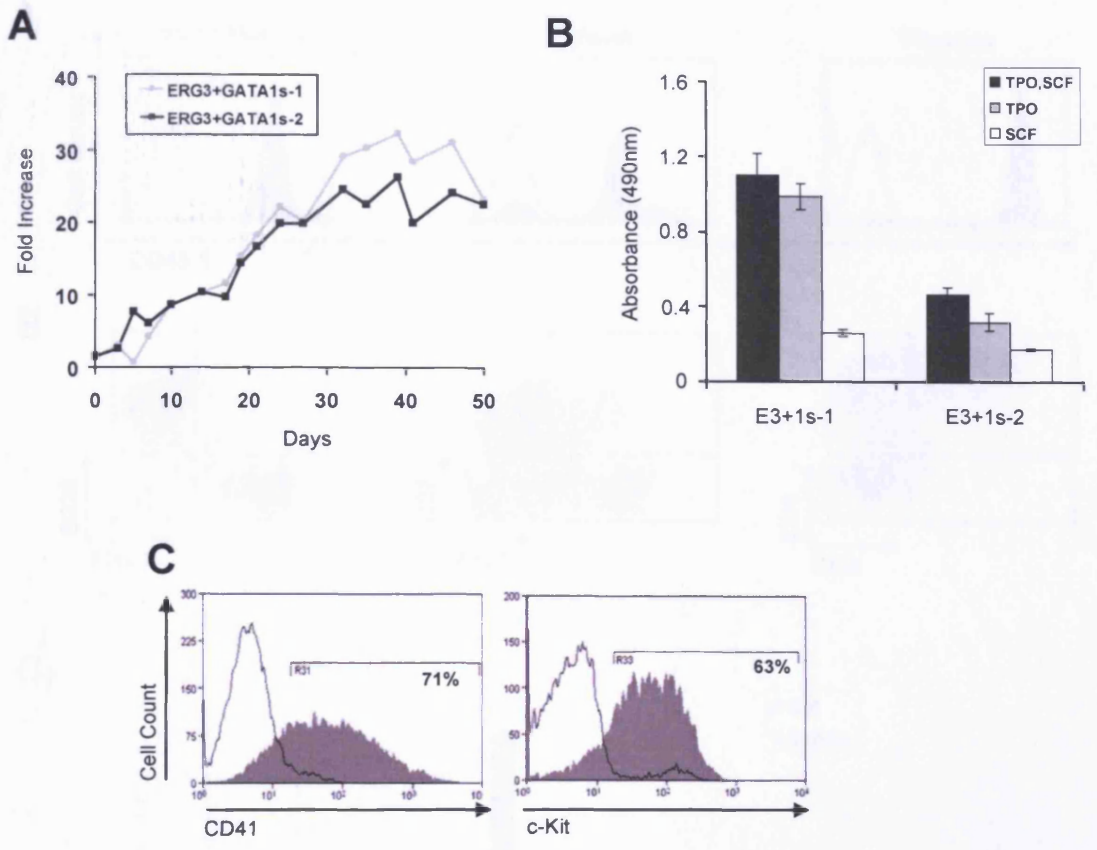


Figure 4.4. Characterisation of ERG-3 + GATA1s immortalised cell lines. A. Fold increase in cell number of immortalised cells expressing both ERG-3 and GATA1s. Single colonies were picked from the third round of plating (independent experiment to Figure 4.3) and cultured in complete DMEM medium supplemented with TPO and SCF. **B.** Bar chart showing the absorbance measured at 490 nm of two ERG-3 + GATA1s (E3+1s) immortalised cell lines grown in the presence of TPO, SCF, TPO alone or SCF alone. The data represent mean and ± S.D. of triplicate cultures. MTS assay was carried out 48 hrs after cytokine addition. **C.** Representative histograms of CD41 and c-Kit expression in immortalised cell lines expressing both ERG-3 and GATA1s. Empty plot represents isotype control and filled plot represent cells positive for the indicated cell surface marker.

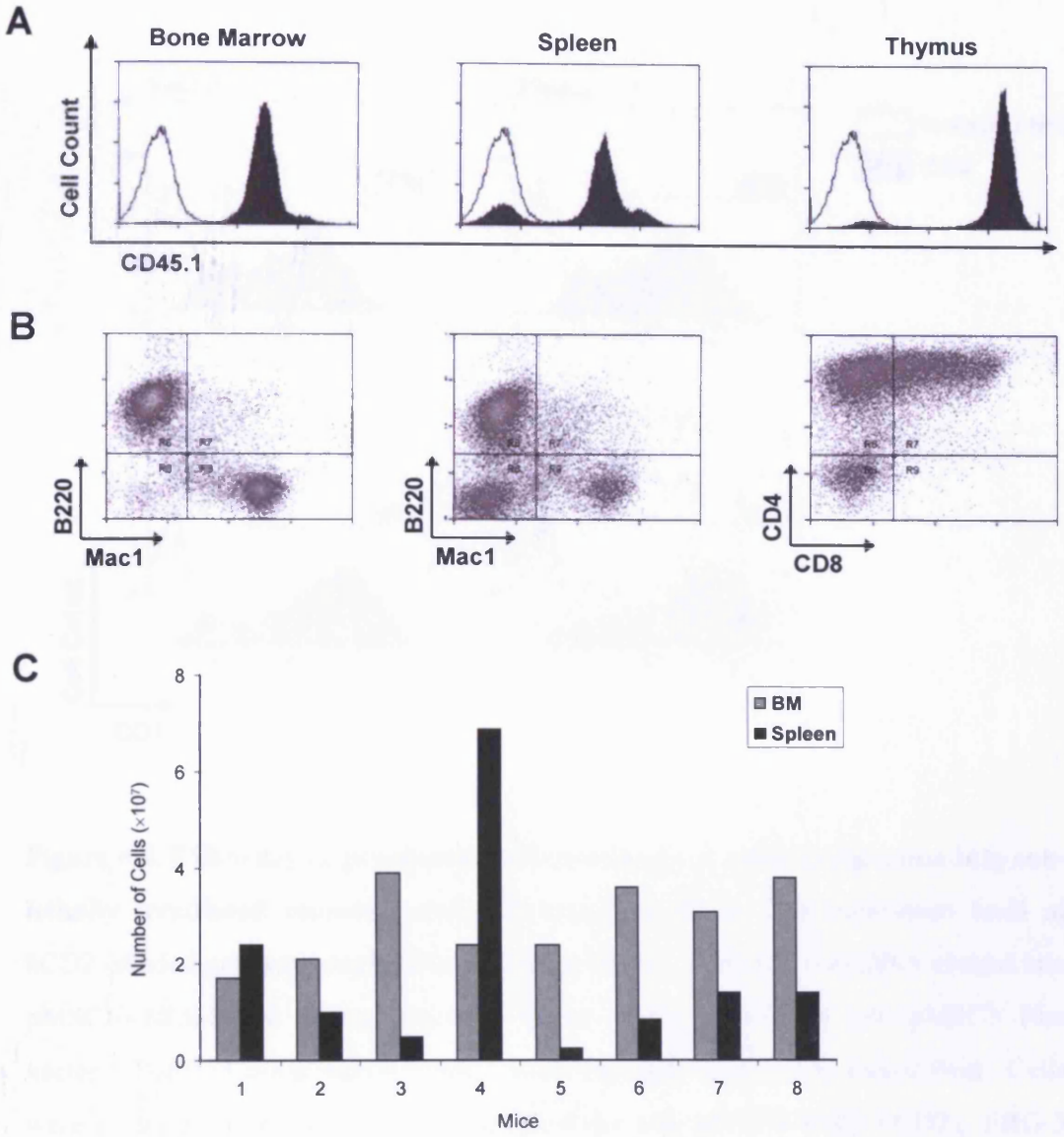


Figure 4.5. ERG-3 + GATA1s immortalised cell lines are not leukaemogenic in NOD/SCID mice. **A.** Histogram showing CD45.1 cells in the bone marrow, spleen and thymus. Empty plot represents isotype control and filled plot represents cells positive for CD45.1. **B.** Dot plots show B220 and Mac1 positive cells in the bone marrow and spleen and CD4, CD8 positive cells in the thymus. **C.** Bar chart representing number of bone marrow (grey bar) cells and splenocytes (black bar) from 8 mice analysed.

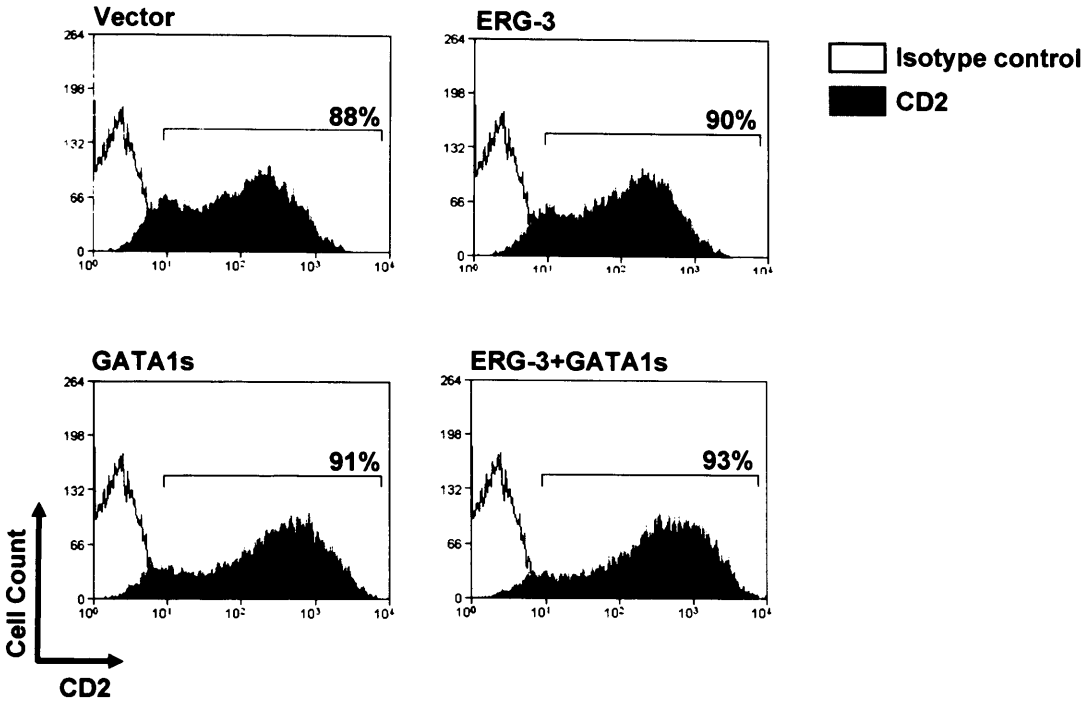


Figure 4.6. Efficiency of progenitor cell transduction prior to injection into sub-lethally irradiated recipient mice. Histogram depicting the expression level of hCD2 (shaded grey) against an isotype control (black line). *ERG-3* cDNA cloned into pMSCV-IRES-hCD2 vector, whereas, *Gata1s* cDNA is cloned into pMSCV-Neo vector. Purified c-Kit$^+$Ter119$^-$ HPCs were isolated from E12.5 foetal liver. Cells were co-transduced with vector (pMSCV-Neo and pMSCV-IRES-hCD2), ERG-3 (pMSCV-*ERG-3*-IRES-CD2 retrovirus and pMSCV-Neo), *Gata1s* (pMSCV-*Gata1s*-neo retrovirus and MSCV-IRES-hCD2) and *ERG-3* + *Gata1*s (pMSCV-*ERG-3*-IRES-CD2 retrovirus and pMSCV-*Gata1s*-neo retrovirus). Infection efficiency was determined 48 hrs following infection by analysing the expression of hCD2. No selection was applied.

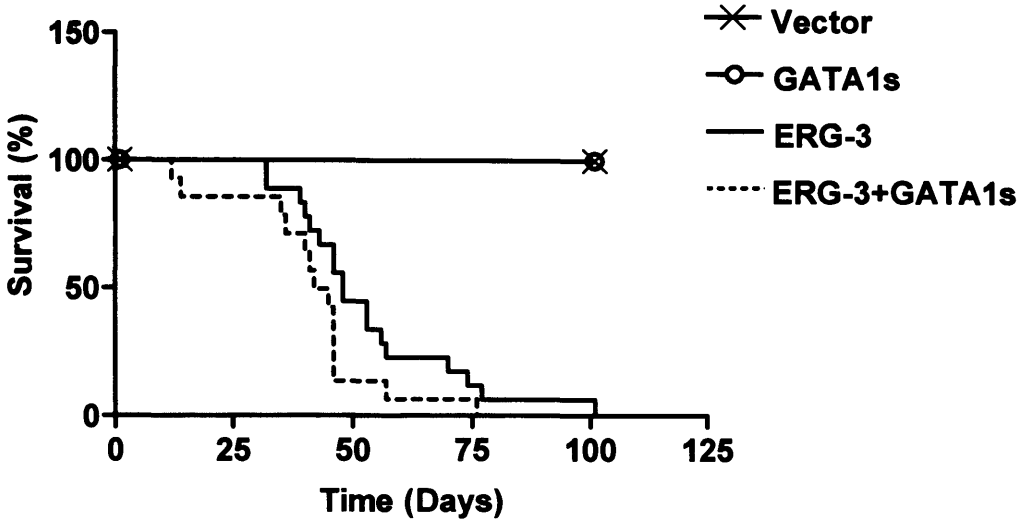


Figure 4.7. Progenitor cells transduced with ERG-3 and ERG-3 + GATA1s induce leukaemia with short latency. Survival curve shown for cohorts of mice injected with cells transduced with indicated viruses (n=10 for vector control and n=5 for GATA1s, n=17 for ERG-3 and n=14 for ERG-3 + GATA1s). Mice were sub-lethally irradiated (6Gγ) 24 hr prior to injection of transduced HPCs. Mice injected with cells expressing GATA1s did not succumb to leukaemia. All mice injected with ERG-3 and ERG-3 + GATA1s succumb to leukaemia but there is no significant difference in the latency (p=0.47).

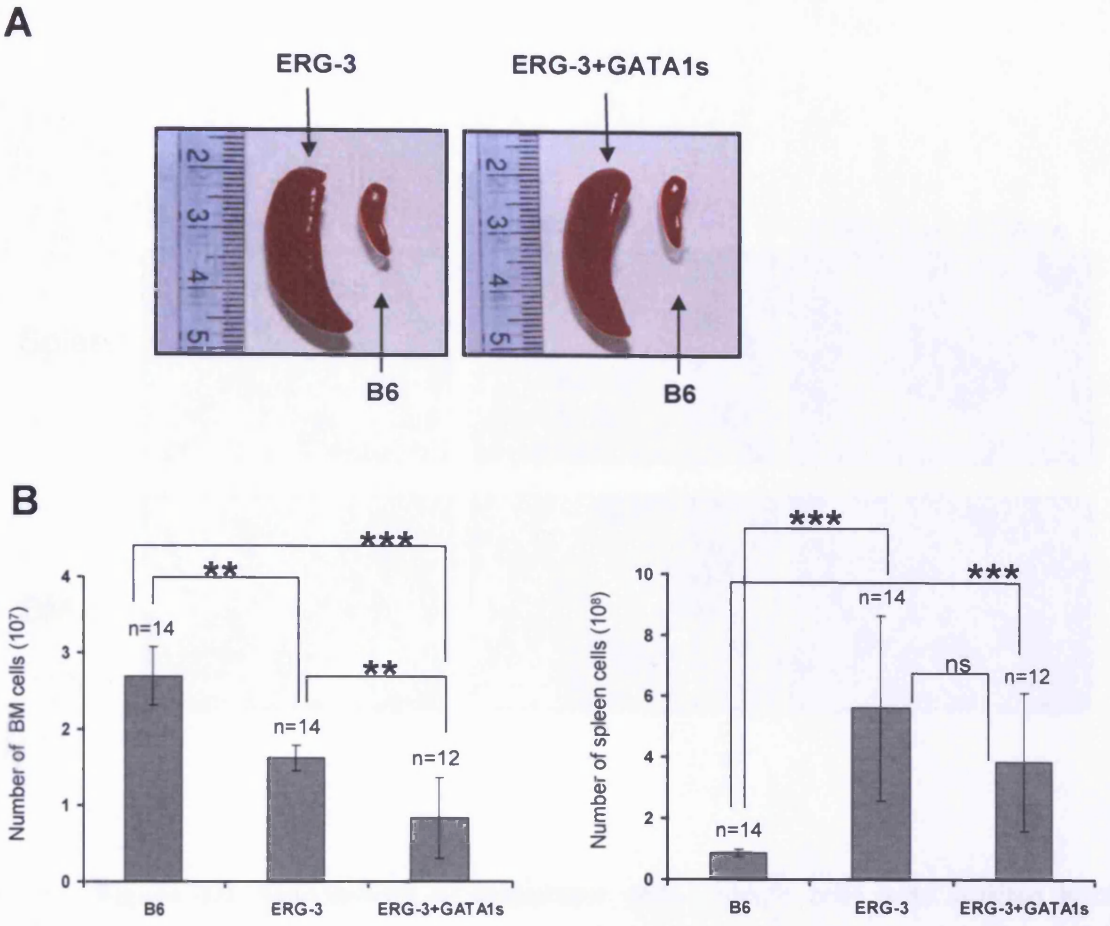


Figure 4.8. Splenomegaly in mice injected with ERG-3 and ERG-3 + GATA1s. **A.** Comparative spleen size in mice injected with ERG-3 and ERG-3 + GATA1s versus wild type B6 mice. **B.** Bar chart showing bone marrow cell number (left panel) and spleen cell number (right panel). Bone marrow cells were reduced in the leukaemic mice (B6 vs ERG-3 p=0.0034, B6 vs ERG-3 + GATA1s p=<0.0001 and ERG-3 vs ERG-3 + GATA1s p=0.031). Splenocytes were significantly increased as compared with wild type mice (B6 vs ERG-3 p=0.0003 and B6 vs ERG-3 + GATA1s p=0.0007). There was no significant difference between splenocytes in mice with ERG-3 as compared with mice transduced with ERG-3 + GATA1s (p=0.81).

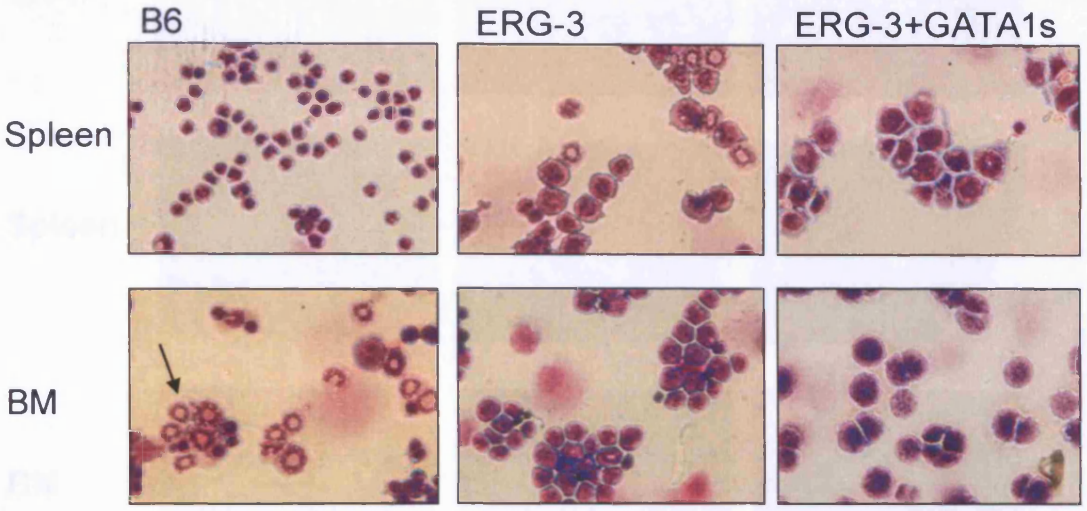


Figure 4.9. Morphology of leukaemic cells. Single cells were isolated from spleen (top panel) and BM (bottom panel) of wild type mice, mice injected with ERG-3 and ERG-3 + GATA1s and stained with Wright-Giemsa (original magnification, ×400). Wild type splenocytes contain mainly lymphocytes whereas megakaryoblasts are observed in the spleen of mice with ERG-3 and ERG-3 + GATA1s. Although neutrophils are detected in the wild type bone marrow (as shown by an arrow), they are replaced with myeloblasts in the leukaemic mice.

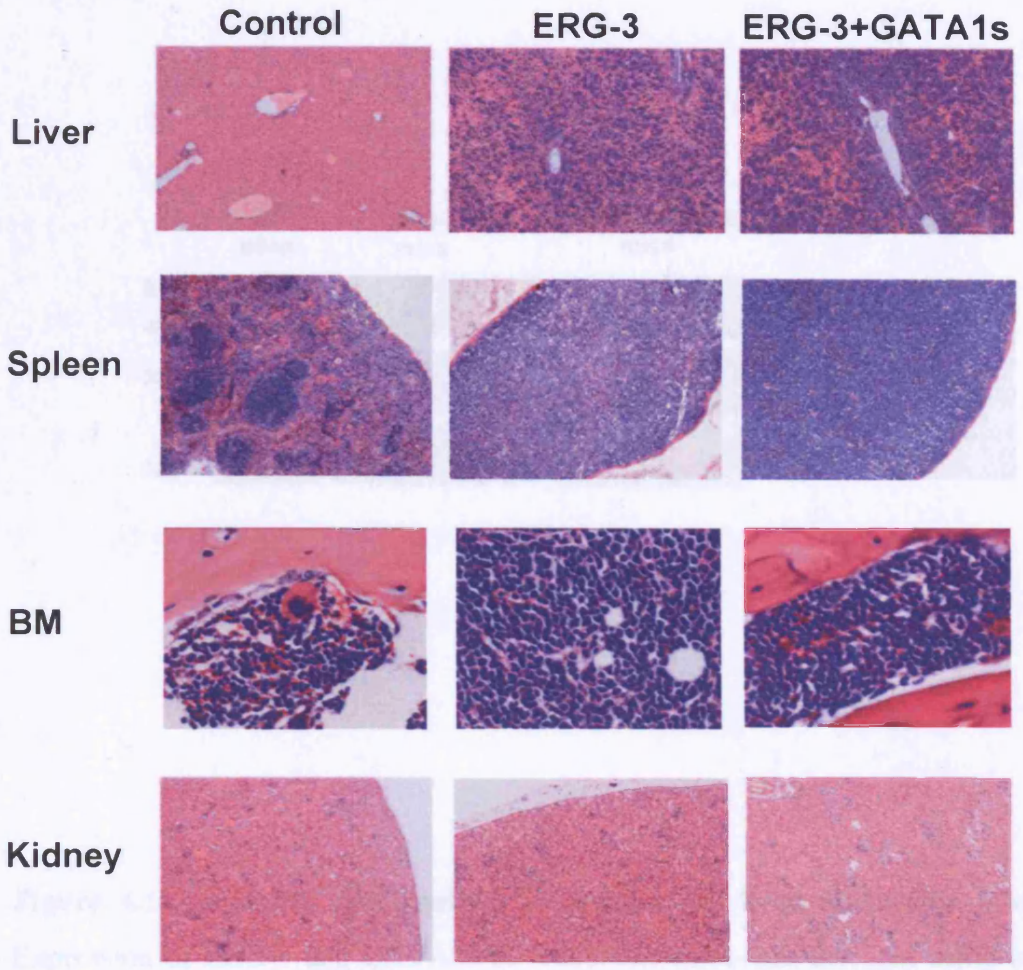


Figure 4.10. Histological analysis of wild type and leukaemic mice. Leukaemic cell infiltration into different organs was determined by haematoxylin and eosin (H&E) staining. Liver, spleen, bone marrow (BM) and kidney were isolated from a control mouse and leukaemic mice. The organs were fixed in formalin and H&E staining was carried out by the department of Pathology at the Institute of Child Health (ICH). No infiltration was detected in kidney, whereas there is a significant infiltration in the liver. There was a replacement of other lineages by the leukaemic blasts in the spleen and bone marrow (original magnification, ×400).

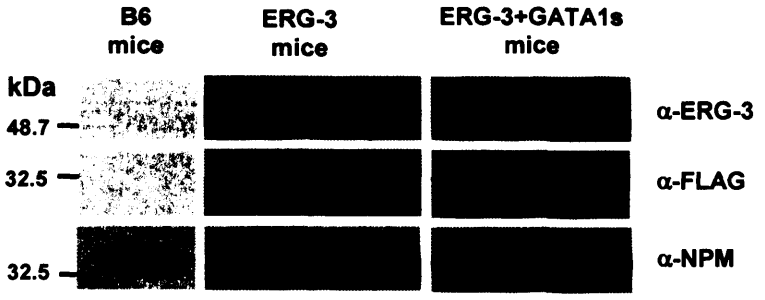


Figure 4.11. Western blot analysis of splenocytes from leukaemic mice Expression of ERG-3 and GATA1s in leukaemic mice was detected using an anti-ERG-3 and anti-FLAG antibodies, respectively, and an anti-NPM antibody was used as a loading control. Protein lysates from spleen were made from two independent mice with ERG-3 and two independent mice with ERG-3 + GATA1s. For a negative control, protein lysates were prepared from B6 wild type mice. The protein lysates were resolved on a 10% polyacrylamide gel.

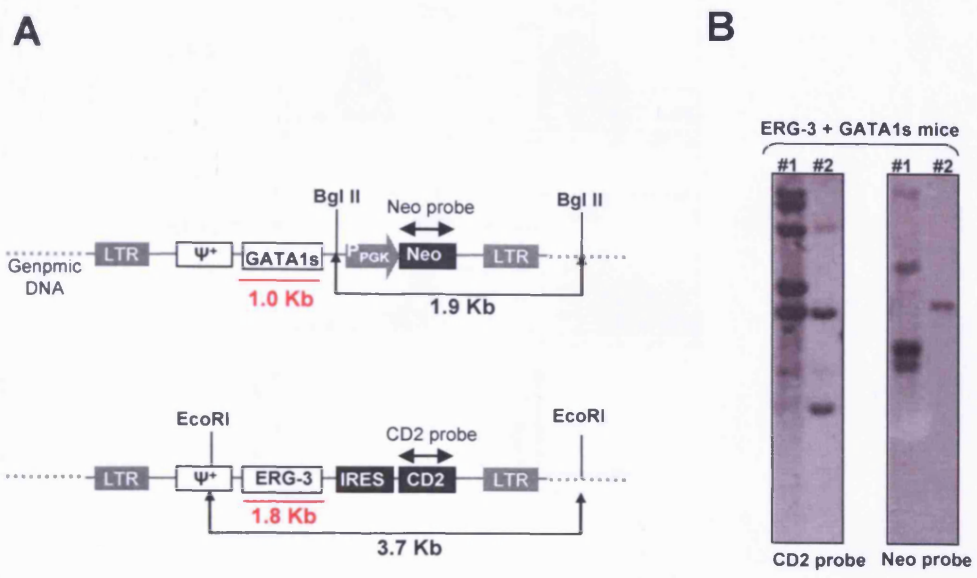


Figure 4.12. Southern blot analysis of genomic DNA isolated form leukaemic splenocytes of ERG-3 + GATA1s mice. A. The indicated neo cDNA fragment probe was used to detect the *Gata1s* provirus following *BglII* digest of genomic DNA. The indicated hCD2 fragment probe was used to detect the *ERG* provirus following *EcoRI* digest of genomic DNA. Both enzymes cut once within the pMSCV retroviral vector which is integrated within the genomic DNA. **B.** CD2 and neo probe were used to identify the pattern of integration for pMSCV-*ERG-3* and pMSCV-*Gata1s* DNA.

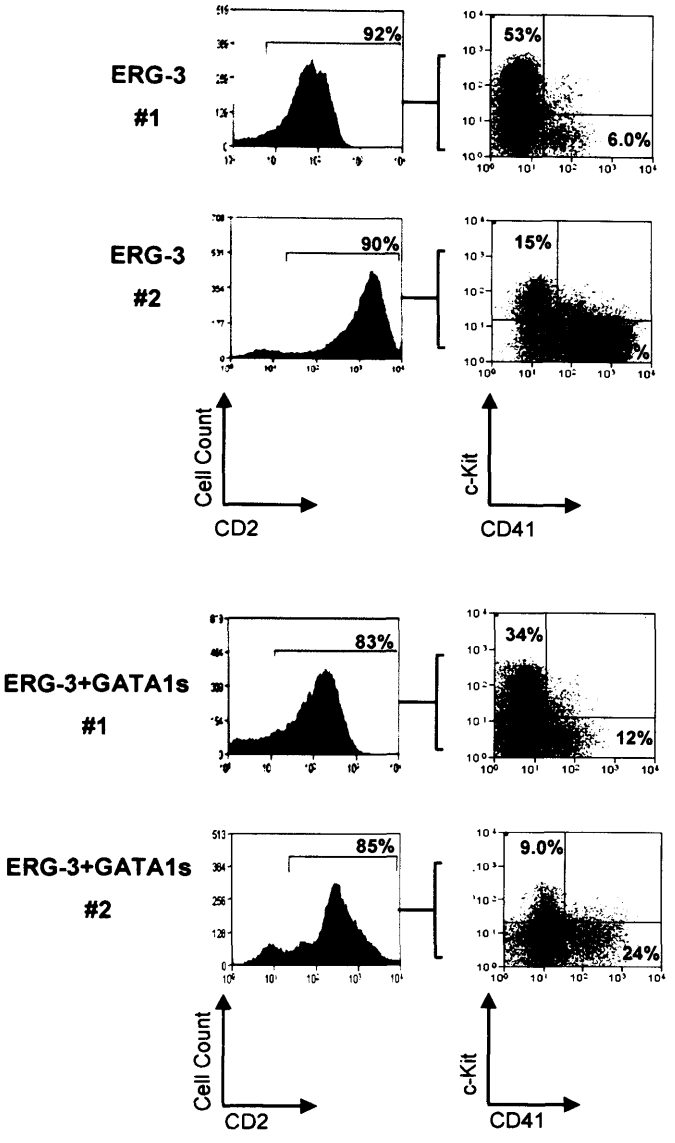


Figure 4.13. Immunophenotypic analysis of leukaemic splenocytes. Flow cytometric analysis of leukaemic splenocytes of representative animals injected with ERG-3 (Top panel) or ERG-3 + GATA1s (Bottom panel). The histograms show the percentage of hCD2 positive cells in the spleen. Dot plots show c-Kit and CD41 expression within the hCD2 positive population. The numbers in the plots represent the percentage of c-Kit positive and CD41 positive cells.

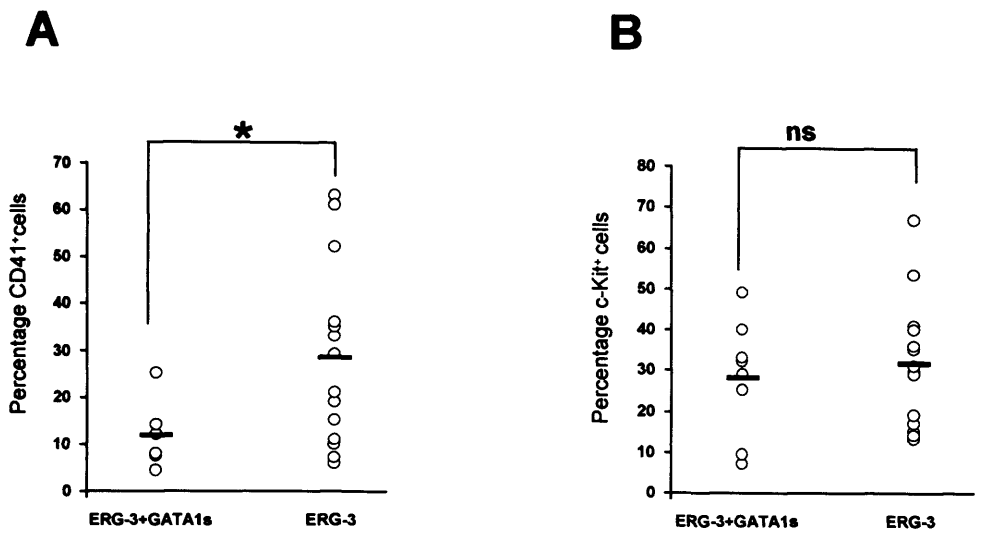


Figure 4.14. Leukaemic cells expressing ERG-3 + GATA1s have a less mature phenotype. Plot shows the percentage of CD41 (**A**) ($p=0.03$) and c-Kit (**B**) positive cells (ns, not significant) within the hCD2 positive splenocytes of mice transplanted with cells expressing ERG-3 only or both ERG-3 and GATA1s.

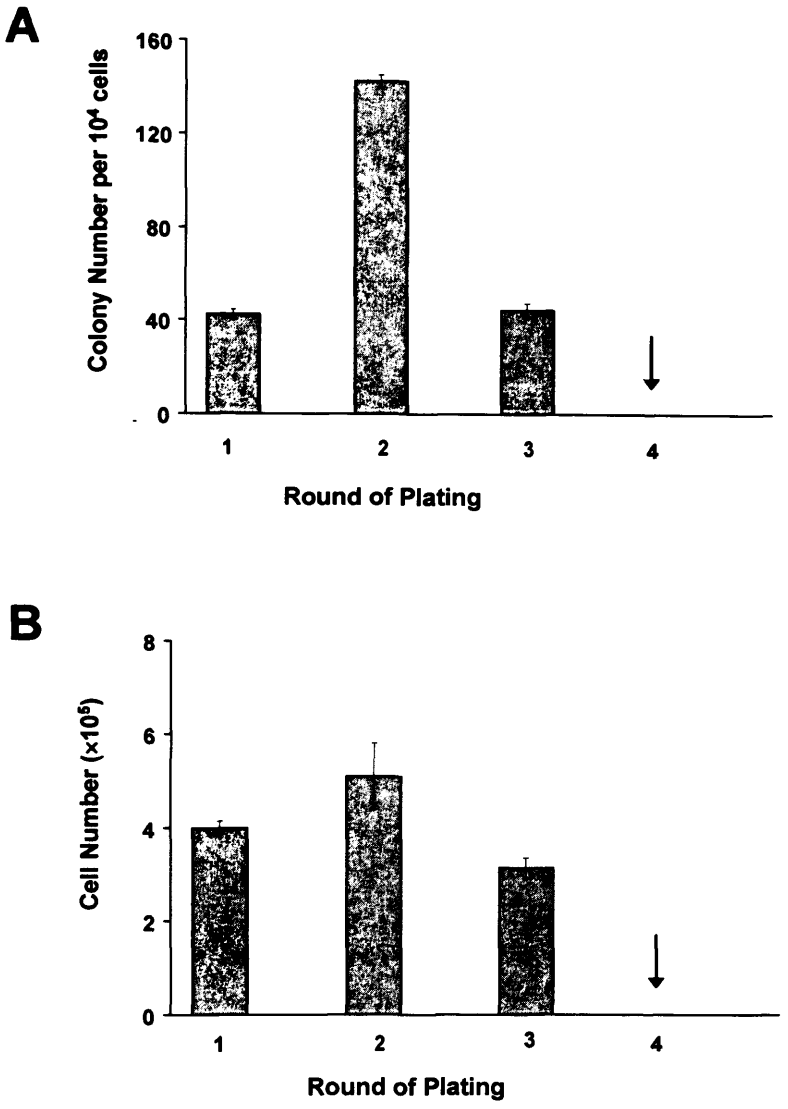


Figure 4.15. Bone marrow cells isolated from ERG-3 leukaemic mice cannot be maintained *in vitro* under myeloid conditions. **A.** Bone marrow was isolated from leukaemic mice transplanted with ERG-3 transduced HPCS. Bone marrow cells were counted and 10^4 cells were cultured in methylcellulose under myeloid conditions. Colonies formed (**A**) at each round of plating were counted and single cells (**B**) were serially plated into the second round and third round after which cells ceased to replate. The arrows indicate absence of colony formation and cell number. The data represents mean and ± S.D. of duplicate cultures.

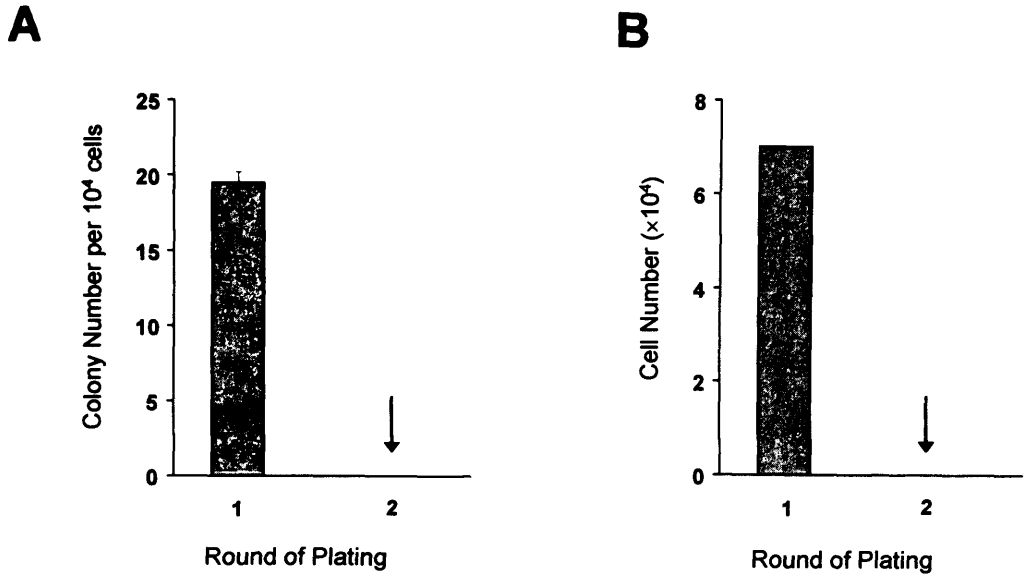


Figure 4.16. Bone marrow cells isolated from ERG-3 leukaemic mice cannot be maintained *in vitro* under megakaryocytic conditions. Bone marrow was isolated from mice transplanted with ERG-3 transduced HPCS. Bone marrow cells were counted and 10^4 cells were cultured in methylcellulose under megakaryocytic conditions in the presence of TPO and SCF. Colonies formed (**A**) and number of cells (**B**) was counted at the first round of plating. ERG-3 transduced cells could not be replated following the first round. The arrows indicate absence of colony formation and cell number. The data represents mean and ± S.D. of duplicate cultures.

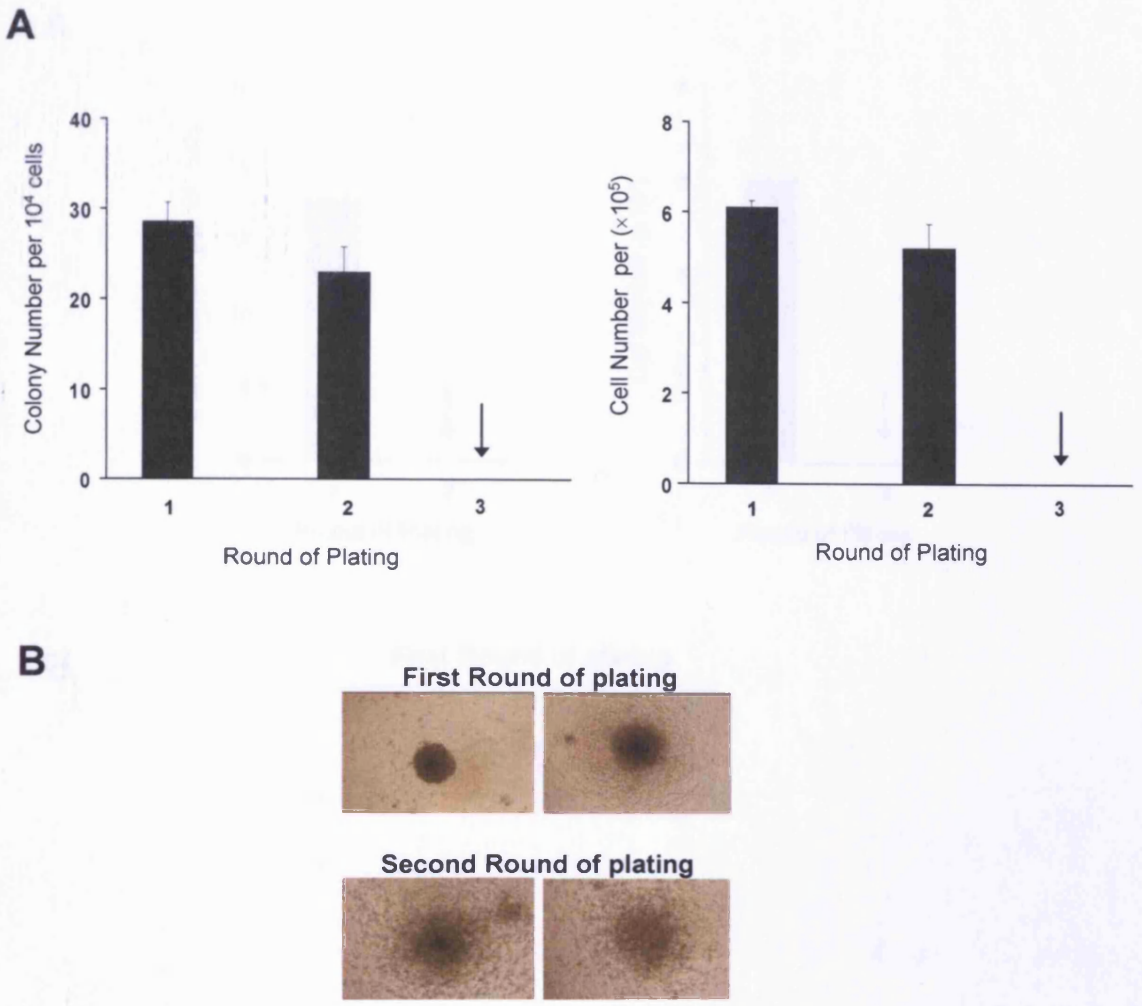


Figure 4.17. Bone marrow cells isolated from ERG-3 + GATA1s leukaemic mice cannot be maintained *in vitro* under myeloid conditions. A. Bone marrow was isolated from mice transplanted with ERG-3 + GATA1s transduced HPCS. Bone marrow cells were counted and 10^4 cells were cultured in methylcellulose under myeloid conditions. Colonies formed at each round of plating were counted and single cells were serially re-plated. ERG-3 + GATA1s transduced cells could not be re-plated following the second round. **B.** Typical morphology of colonies formed at the first and second round of re-plating (original magnification, ×40). The arrows indicate absence of colony formation and cell number. The data represents mean and ± S.D of duplicate cultures.

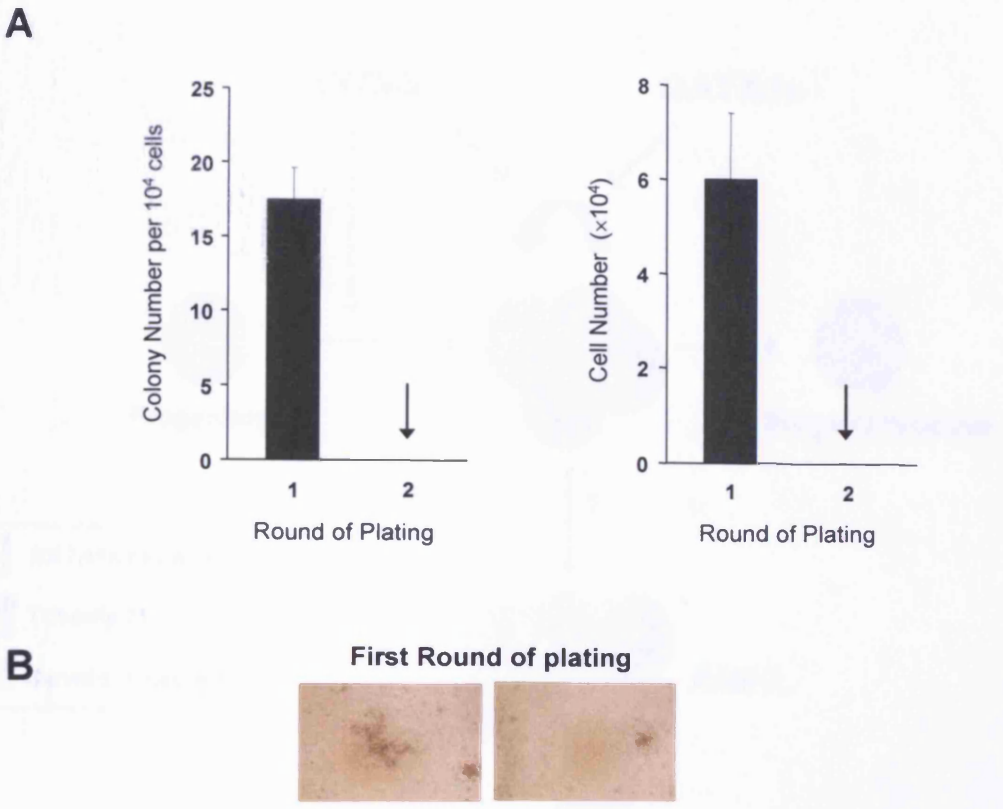


Figure 4.18. Bone marrow cells isolated from ERG-3 + GATA1s leukaemic mice cannot be maintained *in vitro* under megakaryocytic conditions. Bone marrow was isolated from mice transplanted with ERG-3 + GATA1s transduced HPCS. Bone marrow cells were counted and 10^4 cells were cultured in methylcellulose under megakaryocytic conditions in the presence of TPO and SCF. **A.** The number of colonies formed (Left panel) and the number of cells (Right panel) were counted after the first round of plating. ERG-3 + GATA1s transduced cells could not be replated following the first round. **B.** Typical morphology of colonies formed at the first round of re-plating (original magnification, ×40). The arrows indicate the absence of colony formation and cell number. The data represents mean and S.D. of duplicate cultures.

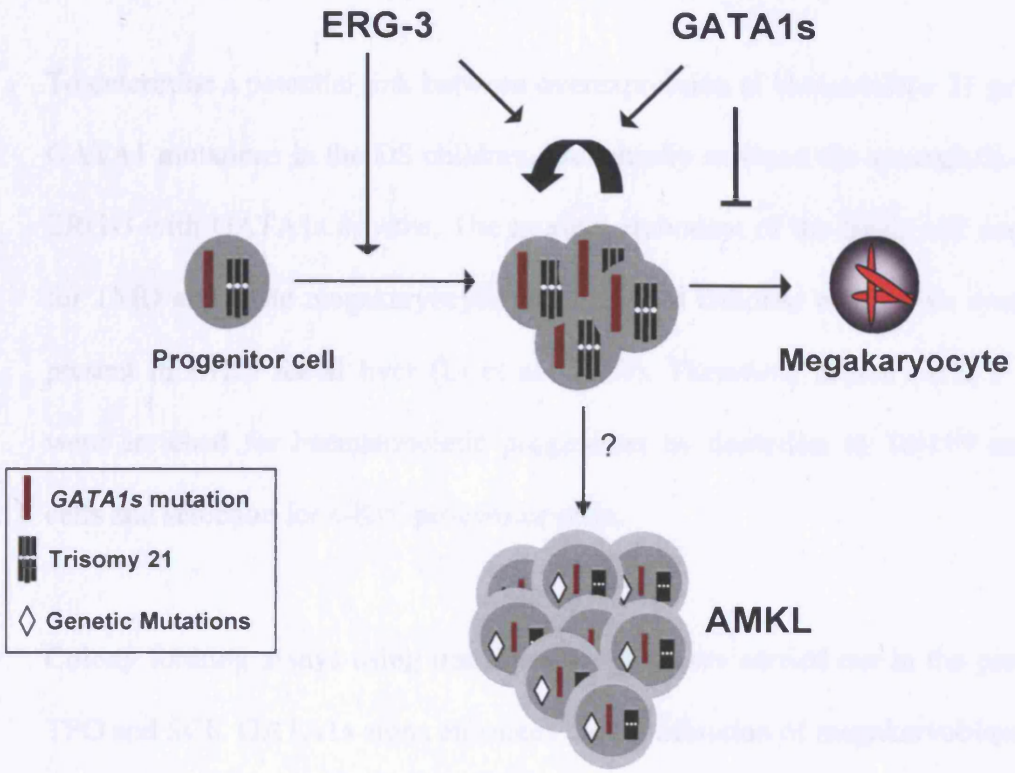


Figure 4.19. Model of leukaemogenesis in DS. Acquisition of chromosome 21 during early embryonic development is believed to be an early initiating event in the pathogenesis of DS. We propose that increased dosage of ERG-3 acts on early progenitor cells to promote megakaryopoiesis and increases the pool of early megakaryocytic cells. Cells trisomic for chromosome 21 also harbour *GATA1s* mutations thus promoting hyperproliferation of megakaryoblasts at the expense of differentiation. Not all DS patients with *GATA1s* mutation develop AMKL, suggesting that ERG-3 and GATA1s are not sufficient for leukaemogenesis and other as yet unidentified mutations are required for AMKL development in these individuals.

4.5 Discussion

4.5.1 ERG-3 collaborates with GATA1s *in vitro*

To determine a potential link between overexpression of chromosome 21 genes with GATA1 mutations in the DS children, we initially assessed the synergistic effect of ERG-3 with GATA1s *in vitro*. The murine equivalent of the target cell responsible for TMD and acute megakaryocytic leukaemia in children with Down syndrome is present in E12.5 foetal liver (Li et al., 2005). Therefore, primary E12.5 FL cells were enriched for haematopoietic progenitors by depletion of Ter119 expressing cells and selection for c-Kit$^+$ progenitor cells.

Colony forming assays using transduced HPCs were carried out in the presence of TPO and SCF. GATA1s alone enhances the proliferation of megakaryoblasts. Since IL-3 also drives megakaryocytic proliferation, this cytokine was omitted from cultures in order to avoid masking of GATA1s activity. HPCs co-expressing ERG-3 and GATA1s could be serially re-plated suggesting that these cells were immortalised. This phenotype was further confirmed by transferring single colonies from methylcellulose into liquid culture containing TPO and SCF, upon which the cells expanded and divided continuously over an extended time course. Immunophenotypic analysis revealed that the ERG-3 + GATA1s immortalized cells had a characteristic immature megakaryoblastic phenotype expressing high levels of c-Kit and CD41 cell surface markers. HPCs expressing GATA1s could not be serially re-plated. Indeed, TMD clones harbouring GATA1s disappear in most DS individuals, strongly suggesting that GATA1s alone is not sufficient to immortalise TMD blasts. The inability of ERG-3 alone to immortalize progenitor cells in this

experiment demonstrates the requirement of ERG-3 expressing cells for IL-3 in addition to TPO and SCF. Furthermore, the data suggest a classic oncogenic co-operativity between GATA1s and ERG-3 that immortalise megakaryoblasts analogous to the megakaryoblastic leukaemia cells found in DS-AMKL.

Cell lines immortalised with ERG-3 + GATA1s were transplanted into NOD/SCID mice to assess their leukaemogenic potential *in vivo*. Following the analysis of the injected mice, the CD45.2 positive immortalised cell lines could not be detected in the bone marrow, spleen or thymus. Factors such as cytokines, chemokines and adhesion molecules are essential for the homing of cells from peripheral injection site to the bone marrow in NOD/SCID mice (Peled *et al.*, 2000). It is possible that the homing capability and responsiveness to specific cytokines of these cell lines have been lost / reduced due to *in vitro* culture of the cells prior to transplantation. Furthermore, the differentiation status of the cells following *in vitro* culture may prevent engraftment in the recipient mice. Our result shows that ERG-3 + GATA1s immortalised cell lines are not leukaemogenic in NOD/SCID mice. To overcome this problem, HPCs freshly co-transduced with ERG-3 + GATA1s were injected directly into sub-lethally irradiated mice.

4.5.2 ERG-3 acts as an oncogene *in vivo*

To determine the leukaemogenic potential of ERG-3 *in vivo*, we transplanted HPCs transduced with *ERG-3* retrovirus into sub-lethally irradiated syngeneic C57BL/6. Our data show that haematopoietic progenitors transduced with ERG-3 rapidly develop leukemia *in vivo*.

The location of the *ERG-3* gene has suggested that its amplification associated with DS-AMKL may be a contributing factor in leukaemogenesis in these children (Bourquin *et al.*, 2006). This study provides evidence that overexpression of the *ERG-3* gene is necessary to exhibit transforming activity. It will be useful to determine what domains of ERG-3 are required for tumourgenesis. ERG-3 may act through gene dosage effect on processes such as megakaryocytic commitment, proliferation and differentiation and may be an important factor for the initiation of leukaemia in DS children. Indeed, over-expression of other candidate genes have exhibited biological characteristics detected in DS. Gene dosage effects have been suggested to be important in non-haematological DS abnormalities. Increased copy number of the *ETS2* is one example of gene dosage effect related to the DS phenotype. ETS2 is overexpressed in brain and fibroblasts from DS patients and increased apoptosis has been detected when *Ets2* gene was overexpressed in murine neuronal cultures (Tymms and Kola, 1994; Wolvetang *et al.*, 2003). In addition, ETS2 overexpression in transgenic mice suggests a potential role for this transcription factor in the skeletal abnormalities associated with DS (Sumarsono *et al.*, 1996). Another promising candidate gene is *DYRK1A*, which encodes a dual-specificity tyrosine phosphorylation-regulated kinase (Guimera *et al.*, 1996). DYRK1A protein is increased in the brains of DS patients as well as in the trisomic T65Dn murine model of DS (Dowjat *et al.*, 2007). It has been proposed that the overexpression of DYRK1A may be a contributing factor to the cognitive difficulties in DS subjects.

ERG involvement in cancer has been highlighted by the involvement of *ERG* gene in chromosomal translocations, which results in the truncation of the ERG-3 N-

terminus. This observation suggests that the N-terminus of ERG may confer a tumour suppressive function. Although increased expression of full length ERG has been associated with poor prognosis of AML, it is unclear if this simply represents the early differentiation stage of the leukaemic blasts or if ERG has a direct leukaemogenic activity. Our data demonstrate for the first time that increased expression of the full length ERG protein causes aggressive leukaemia in mice. It would be interesting to determine the target cell for ERG-3-mediated leukaemogenesis. *In vivo* transplantation assays using ERG-3 expressing HSC, MEP or CMP could determine if ERG-3 is capable of transforming HSCs or a more committed progenitor cell.

4.5.4 ERG-3 collaborates with GATA1s to induce a megakaryoblastic leukaemia *in vivo*

To determine the leukaemogenic potential of ERG-3 and GATA1s *in vivo*, we transplanted HPCs co-transduced with *ERG-3* and *GATA1s* expressing retroviruses into sub-lethally irradiated syngeneic C57BL/6. Haematopoietic progenitors transduced with ERG-3 or ERG-3 + GATA1s rapidly develop leukemia *in vivo* when transplanted.

The *GATA1s* mutation results in the truncation of the GATA1 N-terminal transactivation domain, making the protein transcriptionally less active than GATA1. HPCs expressing GATA1s have ineffective control of proliferation but the differentiation capacity is retained albeit to a reduced level. This suggests a possible explanation for the phenotype observed in that the balance between ERG-3 and GATA1s determines the level of differentiation. Hence leukaemias expressing

ERG-3 alone have higher levels of CD41 expressing megakaryoblasts than those expressing both ERG-3 + GATA1s. This is in agreement with the current data, emphasizing the effect of GATA1s in proliferation. GATA1s is associated with the loss of repression of several GATA1 target genes that have proliferative functions. These genes include, *KIT* and *MYC,* which are downregulated in the presence of GATA1 and are upregulated in DS-AMKL cells harbouring GATA1s and lacking GATA1 (Li et al., 2005; Bourquin et al., 2006). Furthermore, the failure to rescue the hyperproliferative phenotype of GATA1 deficient by GATA1s expression, further supports the role for GATA1s in cell proliferation and also highlighted the importance of the activation domain in the regulation of genes required for megakaryocyte proliferation (Muntean and Crispino, 2005)). This study also showed that GATA1s affects the proliferation and not the differentiation process in megakaryocytes as GATA1s was able to restore differentiation of GATA1 deficient cells. In our system, ectopic expression of GATA1s with ERG-3 results in enhanced proliferation of early progenitor cells in the presence of endogenous levels of GATA1 and the percentage of CD41 positive cells is reduced when both ERG-3 and GATA1s are present. It may be that the expression of proliferative genes, such as *Kit* and *Myc* are higher in ERG-3 + GATA1s leukaemic cells, as compared with ERG-3 leukaemic cells.

Therefore, if fetal HPCs from DS children trisomic for ERG are predisposed towards megakaryopoiesis, those megakaryoblasts which additionally harbour the *GATA1s* mutation will exhibit increased proliferation in the absence of full length GATA1. In addition, GATA1s may affect the function of GATA2, which has been shown to compensate for GATA1 in early megakaryopoiesis (Chang et al., 2002). In

this regard, GATA2 expression was shown to be upregulated in DS-AMKL cells as compared with non DS-AMKL (Bourquin et al., 2006).

The high expression levels of ERG in patients with AMKL, highlights its importance in leukaemic development. It is therefore possible that the overexpression of ERG-3 could contribute to leukaemic transformation by inducing a growth advantage to HPCs, a phenotype that may be further exacerbated in the context of GATA1s. Although most DS patients with trisomy 21 and the GATA1 mutation develop TMD in the first year of life, most undergo spontaneous remission. Therefore, it is likely that the acquisition of additional genetic events is required for leukaemic development. We propose that the increased expression of ERG in fetal haematopoietic progenitors trisomic for chromosome 21 promotes megakaryopoiesis. This event co-operates with the enhanced proliferation associated with the *GATA1s* mutation (**Figure 4.19**) giving rise to a clone that serves as a target for additional mutations of leukaemic transformation.

Somatic mutations in the *JAK2* (Janus Kinase) and *JAK3* genes are proposed to account for the additional mutations required for the transformation of TMD to AMKL in DS patients. Indeed, mutations in the *JAK2* and *JAK3* tyrosine kinase have been detected in AMKL cell lines and in some DS-AMKL patients, respectively (Mercher *et al.*, 2006; Walters *et al.*, 2006). These mutations cause constitutive activation of STAT-5 (Signal transduction and activator of transcription) (Spiekermann *et al.*, 2003), and in common with activating mutations in tyrosine kinases, such as in *KIT* and in internal tandem duplication (ITD) and activation loop mutations in *FLT3*, cause constitutive activation of STAT-5 (Signal

transduction and activator of transcription) (Spiekermann et al., 2003). The activation of the JAK-STAT signaling pathway is important in early stages of megakaryocytic differentiation and decreased activation is required for terminal maturation. Therefore, *JAK* mutation associated with DS-AMKL may result in a block in terminal differentiation, causing abnormal accumulation of megakaryoblasts. ERG-3 and GATA1s may work in concert with *JAK* mutations to induce AMKL in DS individuals. Furthermore, mutations in *FLT3*, *KIT* and *RAS*, confer a selective advantage to HSCs, and may work in conjunction with ERG-3 and GATA1s to induce AMKL. Other contributing factors may be activation of pathways such as Notch, Sonic hedgehog (shh) and Wnt signaling. The identification of secondary genetic events necessary for the progression into AMKL would allow the application of therapeutic strategies at the TMD stage of disease to prevent leukaemic transformation. For example, the use of tyrosine kinase inhibitors may be good therapeutic agents, if indeed activating tyrosine kinases provided the additional genetic factor.

Although in humans, trisomy 21 and GATA1s are not sufficient for leukaemic transformation, over-expression of ERG-3 in the presence of GATA1s was enough to induce leukaemia in our murine model. It is likely that this is due to the difference in the expression levels of ERG-3. The level of ERG expression achieved in our retroviral transplantation studies may well be higher than the expected 1.5 increase in ERG expression in foetal liver HPCs of DS embryos. Therefore, it is quite likely that other chromosome 21 genes cooperate with *ERG* in the pathogenesis of DS AMKL. These include *BACH1* and *SON* genes, which were shown to be overexpressed in DS-AMKL as compared with non DS-AMKL

patients. The negative regulation of megakaryocytic differentiation by BACH1 and increased proliferation by SON may be additional events that cooperate with ERG and GATA1s to induce transformation. Our model does not rule out a role for other chromosome 21 genes in DS AMKL. Recent work has argued against a requirement for *RUNX1* (Kirsammer et al., 2008) but has emphasized the possible involvement of *ETS2* in DS-AMKL (Stankiewicz and Crispino, 2007; Ge *et al.*, 2008). High expression levels of ETS2 in megakaryoblastic leukaemias, has suggested its role in DS-AMKL development. Furthermore, in common with ERG-3, ectopic expression of ETS2 in K562 cells induced a lineage switch, from erythroid to megakaryocytic development (Ge et al., 2008). In accordance with our data, Stankiewicz and Crispino have shown that ERG induces megakaryopoiesis and it synergises with GATA1s to enhance colony formation (Stankiewicz and Crispino, 2007).

4.5.5 Primary leukaemic cells were not transplantable

Several factors could account for the failed transplantation of cells into secondary recipients. Acute leukaemias can be defined by any combination of either the accumulation of at least 20% blasts in spleen or bone marrow, lethality to primary animal with latency of 4 weeks or less and transplantable to normal, or sub-lethally irradiated secondary mice (Kogan *et al.*, 2002). The leukaemias detected in our system fit the first two criteria for defining myeloid leukaemias and are therefore classed as acute leukaemias even though they lack transplantable activity. There are currently no reports of AMKL cells from DS patients being xeno-transplanted into immunodeficient mice. The low availability of patient samples may be a limiting factor in performing these experiments. Alternatively, it is possible that AMKL cells derived from DS patients are not transplantable. The frequency of LSC could also

contribute to the effectiveness of transplantation. In our study, only 10^6 unsorted primary leukaemic cells were transplanted into secondary recipients. As the rarity and the nature (HSC or committed progenitor) of the putative LSC is not known in our system, the total cell number should be increased for secondary transplantation in the future studies. To investigate whether leukaemia originates from the HSC pool or from the committed progenitor pool, cells can be purified based on cell surface markers that enrich for normal stem cells or progenitors. These cell fractions can be subsequently transplanted into recipient mice, which would allow us to identify the cell population responsible for leukaemia.

In our system, the acquisition of mutations in genes such as *Jak3* or other tyrosine kinases (*Flt3/Kit)* may be essential for the maintenance of leukaemic stem cells or progenitor cells, which is required for serially transplantable leukaemia. *JAK3* mutation, associated with AML and AMKL is one example of a genetic mutation, which was shown to give rise to AMKL phenotype in a murine model (Walters et al., 2006). However, the disease was not transplantable and it was proposed that cooperating mutations with the *GATA1s* mutation in the background of trisomy 21 is required for full AMKL transformation.

Another contributing factor may be the differentiation status of the ERG-3 and ERG-3 + GATA1s transformed cell. Different cellular populations have varied homing capacities. For example, xeno-transplantations experiments show that primitive $CD34^+CD38^-$ progenitors, which have long term repopulating capacity, homed to the BM and spleen whereas more mature progenitors $CD34^+CD38^+$ were not able to home (Kerre *et al.*, 2001). In addition, Bonnet and Dick have shown that

only leukaemic blasts with phenotypic characteristic similar to normal HSCs were transplantable in NOD/SCID mice (Bonnet and Dick, 1997). However, other published data show that more mature cells can be a target of leukaemic transformation (Cozzio et al., 2003; Huntly et al., 2004). Based on these data, it is possible that the target cell for ERG-3 and GATA1s is a more committed progenitor cell such as an MEP. Our data suggests that the accumulated leukaemic cells have an early megakaryoblastic phenotype as shown by the expression of CD41 as well as cellular morphology.

Whilst overexpression of ERG-3 and GATA1s are sufficient to transform progenitor cells, other activating mutations that confer a self renewal advantage are required for transplanting the leukaemic cells. Targeting of the leukaemic stem cells by chemotherapeutic drugs is currently a major obstacle in treatment of leukaemias. Current therapies for leukaemia typically target proliferating cells; however LSCs are quiescent which makes them less responsive to such agents. If the target cell in DS-AMKL is a more differentiated progenitor cell, it may explain why these patients have a very good responsiveness to chemotherapy as compared with non DS-AMKL individuals. This may be also due to increased expression of the chromosome 21 gene *SOD* (superoxide dismutase), which promotes apoptosis. The increased expression of another chromosome 21 gene, *CBS* (cystathionine β-synthetase), which catalyses the condensation of serine and homocystein to from cystathionine, has been correlated with increased sensitivity to cytosine arabinoside (ara-C)(Taub *et al.*, 1999; Ge *et al.*, 2003).

Chapter 5

Results

Role of NPM-MLF1 associated with MDS

Chapter 5: Role of NPM-MLF1 associated with MDS

MDS is characterised by abnormal differentiation and development of myeloid cells, erythrocytes and megakaryocytes. There are a number of chromosomal translocations and genetic mutations associated with MDS, one of which is the chromosomal translocation that generates the *NPM-MLF1* fusion gene. In order to investigate the potential role of NPM-MLF1 in inducing MDS we carried out *in vitro* colony forming assays.

5.1 Generation of retroviral constructs

Human *NPM-MLF1*, *NPM* and *MLF1* cDNAs were cloned into the pMSCV based retroviral vector, containing a neomycin resistance gene (**Figure 5.1A**). The *NPM-MLF1* and *NPM* constructs contain a FLAG epitope at the N-terminus, whereas the *MLF1* contains a Myc epitope at the C-terminus. Human LinXE packaging cell lines were tranfected with retroviral constructs and total lysates were prepared 48 hours after transfection. Immunoblotting with an anti-Flag antibody revealed the presence of 48 kDa NPM-MLF1 protein and a 37 kDa NPM protein. An anti-Myc antibody was used to detect the expression of an approximately 31 kDa MLF1 protein. An antibody against α-tubulin was used as a loading control (**Figure 5.1B**).

6.2 NPM-MLF1 affects myeloid differentiation

We have used mouse BM-derived progenitor cells isolated from 5-FU treated mice to examine the role of NPM-MLF1 in the pathogenesis of MDS. BM cells were transduced with vector or *NPM-MLF1* retrovirus and cells were cultured in

methylcellulose media supplemented with GM-CSF to promote proliferation and differentiation of HPCs along the myeloid lineage. Due to the presence of the neomycin resistance gene in the retroviral vectors we were able to select for infected cells in G418 in the initial round of plating. After 7 days of culture at 37°C, colonies were counted and cells were harvested for flow cytometry. The ability of NPM-MLF1 expressing cells to self-renew and form colonies in culture was also examined. Vector transduced cells formed approximately equal numbers of colonies in the first two rounds, whereas there was a significant increase in the number of colonies formed by NPM-MLF1 transduced cells at the second round of plating. Cells transduced with vector alone failed to replate following the third round of plating. There was a marked reduction in colony formation by NPM-MLF1 expressing cells in the third and fourth round after which cells ceased to replate further (**Figure 5.2A**). There was also a distinct difference in the morphology of colonies formed by NPM-MLF1 and vector. Colonies formed by cells transduced with vector alone were typically large and sparse in contrast to small, compact colonies formed by NPM-MLF1 expressing cells (**Figure 5.2B**).

The harvested cells were analysed for cell surface expression of myeloid differentiation markers Mac1 and Gr1 and the early progenitor cell marker c-Kit. In the first round of plating, the percentage of cells expressing Mac1 and Gr1 was reduced in NPM-MLF1 expressing cultures. Furthermore, there was a two-fold increase in the number of c-Kit$^+$ progenitor cells (**Figure 5.3A**). In the subsequent rounds, 90% of cells from both cultures expressed c-Kit (**Figure 5.3B**). These results show that NPM-MLF1 causes expansion of early progenitor cell and blocks myeloid differentiation when expressed in BM progenitors.

Although, MDS is primarily a disease of elderly individuals, many DS-AMKL children are predisposed to MDS development prior to leukaemic progression within the first 3 years of life. It is believed that the initiating events that contribute to TMD and AMKL occur during embryogenesis. It is therefore possible that genetic changes required for MDS development occur at very early stages during development. We therefore targeted NPM-MLF1 to progenitor cells isolated from foetal liver to compare the effect of the fusion protein and its potential function in progenitor cells isolated from adult bone marrow. NPM-MLF1 expression was targeted to FL-derived HPCs by retroviral infection. FL cells were enriched for haematopoietic progenitors by depletion of Ter119 expressing cells and selecting for c-Kit$^+$ progenitor cells. Cells were transduced with *NPM-MLF1* retrovirus and the full length *NPM* and *MLF1* retroviral constructs. There was an increase in the number of colonies formed at the third round of plating with NPM-MLF1 transduced cells and this was further increased at the fourth round of plating, after which a reduction in colony formation was observed. The vector, NPM and MLF1 transduced cells formed similar number of colonies, however, these cells did not replate past the sixth round, whereas NPM-MLF1 expressing cells formed colonies in the seventh round (**Figure 5.4A**). There was no significant difference in the total number of cells between each culture even though the number of colonies formed by NPM-MLF1 cells was much higher (**Figure 5.4B**). This is due to the type of colonies detected in each culture, with the NPM-MLF1 colonies being small and compact. Analysis of cells harvested from the first round of plating revealed an 8-fold reduction in the percentage of Mac1 positive cells, expressing NPM-MLF1 compared with vector alone (**Figure 5.5**). The fold reduction in Mac1 expression detected in NPM-MLF1 transduced cells as compared to empty control cells were

demonstrated in four independent infection experiments **(Figure 5.6).** These data demonstrate that NPM-MLF1 may cause ineffective differentiation of myeloid cells.

Cells from third round of plating were put in liquid culture in the presence of SCF, IL-3 and IL-6 to establish if NPM-MLF1 can immortalise progenitor cells. NPM-MLF1 expressing cells grew at a much higher rate than cells transduced with vector, NPM or MLF1 retrovirus (**Figure 5.7**). However, NPM-MLF1 expressing cells did not immortalise progenitor cells as the cells failed to grow past 47 days.

6.3 NPM-MLF1 does not affect megakaryocytic development

In order to examine the role of NPM-MLF1 in megakaryopoiesis, E12.5 FL-derived HPCs were transduced with *NPM-MLF1* retrovirus and cultured in methylcellulose medium in the presence of TPO and SCF in order to promote megakaryopoiesis. As a control, progenitor cells were also transduced with full length *NPM* or *MLF1* retrovirus. Transduced cells were selected for using G418 in the first round of plating. Following 10 days in culture, colonies were harvested and cells were analysed by flow cytometry. The number of colonies generated by cells expressing the different retroviruses was similar in the first and second round of plating. At the third round of plating, however, the number of colonies formed by NPM expressing cells was increased 3-fold, whereas the colony number of NPM-MLF1 decreased **(Figure 5.8A and B)**.

In the first round of plating, the percentage of CD41 positive progenitor cells transduced with NPM-MLF1 was similar to the cells transduced with vector alone or MLF1 retroviral vector. In contrast, there was an approximately 30% increase in

the number of immature megakaryocytes as measured by CD41 cell surface marker (**Figure 5.9A**). Although the CD41 positive cells decrease in the subsequent round of plating, the CD41 expression on cells overexpressing NPM remained high in comparison with the other viruses (**Figure 5.9B**). Almost all of the cells in the first round of plating were c-Kit positive progenitor cells (**Figure 5.9C**). Two additional infection experiments further suggested that full length NPM promoted megakaryocyte development. An increase in the CD41 positive cells were detected with progenitor cells transduced with NPM (**Figure 10**). These data suggest that NPM but not NPM-MLF1 affects the maturation of megkaryocytes.

5.4 *In vivo* transplantation

There are currently several murine models for MDS. By performing reconstitution experiment using cells transduced with NPM-MLF1, we may be able to gain better understanding of the exact role of the chimeric protein in MDS. To further investigate the potential role of NPM-MLF1 in MDS, E12.5 FL-derived HPCs transduced with NPM-MLF1 were transplanted into sub-lethally irradiated mice. The *NPM-MLF1* and *NPM* cDNA were cloned into the pMSCV-IRES-CD2 retroviral vector. The infection efficiency was measured by the expression level of hCD2 gene (**Figure 5.11A**). Infection efficiencies were typically 70%, 55% and 40% for cells expressing vector, NPM and NPM-MLF1, respectively (**Figure 5.11B**).

Donor and the recipient cells can be identified due to the differential expression of allotypic cell surface markers. The transduced progenitor cells express CD45.2 whereas the recipient cells express the CD45.1 antigen. As the infection efficiency

was typically low for NPM-MLF1, the viral titre for the retroviruses was measured by infecting mouse 3T3 fibroblasts with the serial dilutions of retroviral supernatant. The infection efficiency was measured 48 hrs after transduction by measuring the level of hCD2 expression (**Figure 5.12A**). The results indicate that NPM-MLF1 retrovirus produces lower viral titre than *NPM* retrovirus (**Figure 5.12B**).

The transduced cells were injected into sub-lethally irradiated mice and peripheral blood was analysed for reconstitution, 6 weeks after injection. The percentage of donor $CD45.2^{+}CD2^{+}$ cells expressing NPM-MLF1 was extremely low (2.0%) compared with cells expressing NPM (21%) or vector (23%) (**Figure 5.13A**). The small population of cells that were CD2 positive and CD45.2 negative may be erythrocytes or megakaryocytes as these cells are thought to lack CD45 expression (Forsberg et al., 2006). Poor contribution to reconstituted cells was detected in all 4 mice that were injected with NPM-MLF1 expressing progenitor cells (**Figure 5.13B**). Long-term reconstitution was examined by analysing BM cells and splenocytes a year and two months after injection. The percentage of $CD45.2^{+}CD2^{+}$ cells was reduced in comparison to the analysis at 6 weeks post transplantation (**Figure 5.14**).

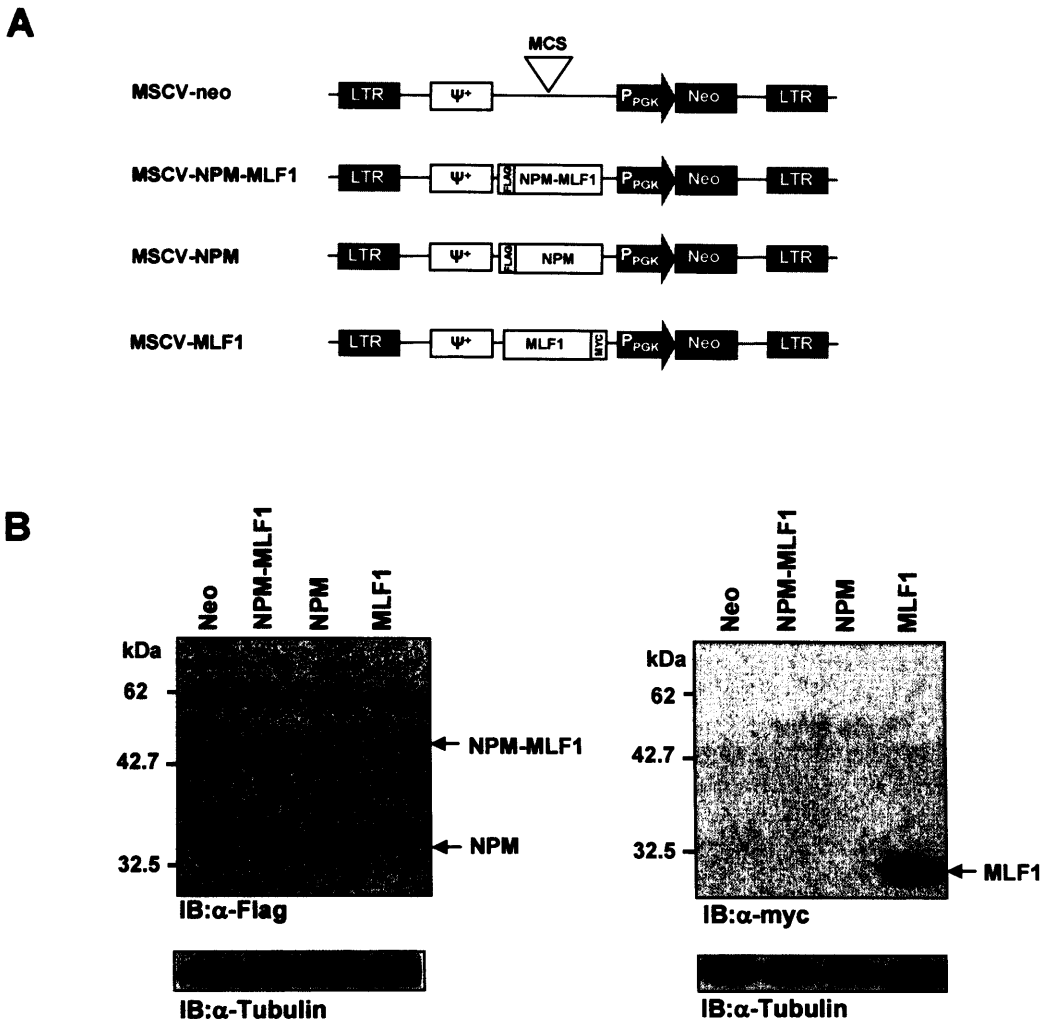


Figure 5.1. Retroviral constructs used to transduce primary HPCs. A. Human *NPM-MLF1*, *NPM* and *MLF1* were cloned into the pMSCV retroviral based vectors. MCS (multiple cloning site). **B.** Immunoblotting to detect the expression of FLAG-tagged *NPM-MLF1*, FLAG-tagged *NPM* and *MYC*-tagged MLF1 proteins. Non-specific bands are indicated by an asterisk. LinXE cells were transfected with indicated retroviral constructs and total lysates were prepared 48 hours after transfection.

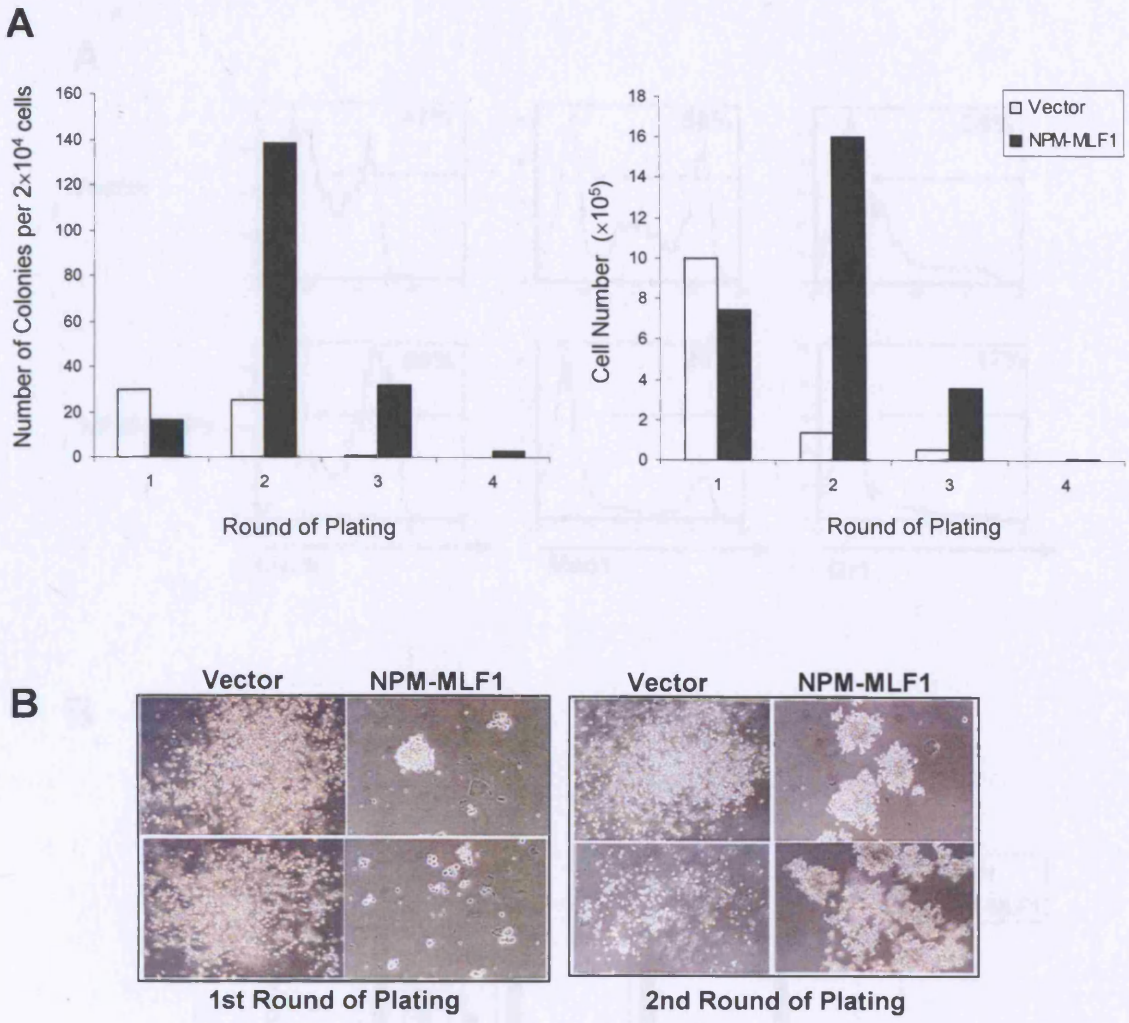


Figure 5.2. BM derived HPCs from 5-FU treated mice: NPM-MLF1 enhances self renewal potential of HPCs under myeloid conditions. A. Bar chart showing number of colonies formed (left panel) and number of cells (right panel) at each round of plating. C57BL/ 6 mice were injected with 5-FU and bone marrow cells were extracted 5 days after treatment. Cells were transduced with empty vector and *NPM-MLF1* retrovirus and cultured in 3434 methylcellulose media in the presence of GM-CSF. Transduced cells were incubated at 37°C for 7 days after which the number of colonies formed and the total cell number were counted. **B.** Morphology of colonies from the second and third round of plating (original magnification ×100). Colonies were counted as clusters of more than 50 cells.

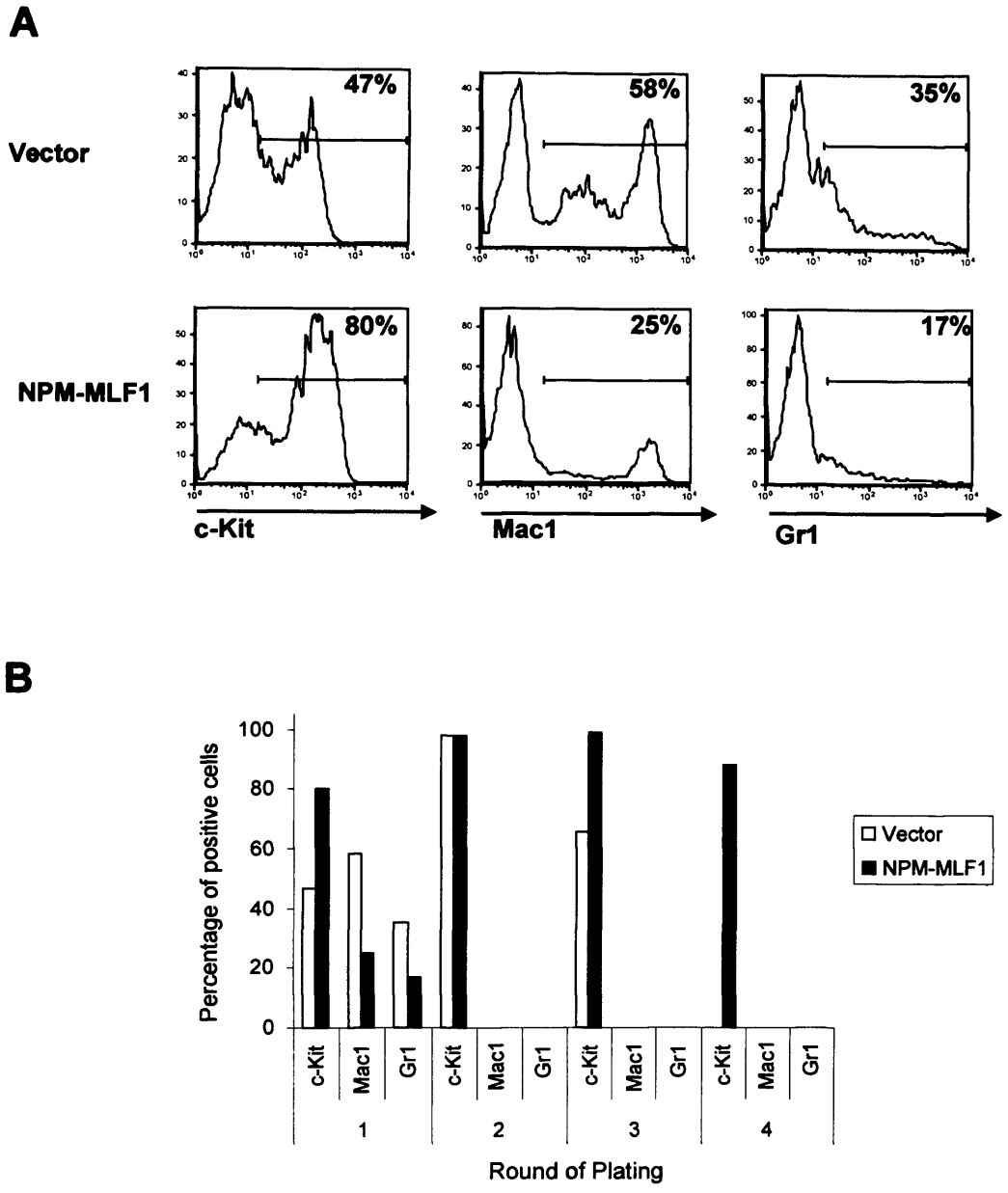


Figure 5.3. BM derived HPCs from 5-FU treated mice: NPM-MLF1 impairs myeloid differentiation. A. Histogram depicting the percentage of c-Kit, Mac1 and Gr1 positive cells. Single cells from the first round of plating were analysed by flow cytometry for cell surface expression of c-Kit, Mac1 and Gr1. The number in the plots represent the percentage of positive cells. **B.** Bar chart representing the percentage of c-Kit, Mac1 and Gr1 positive cells from the first, second, third and fourth round of plating.

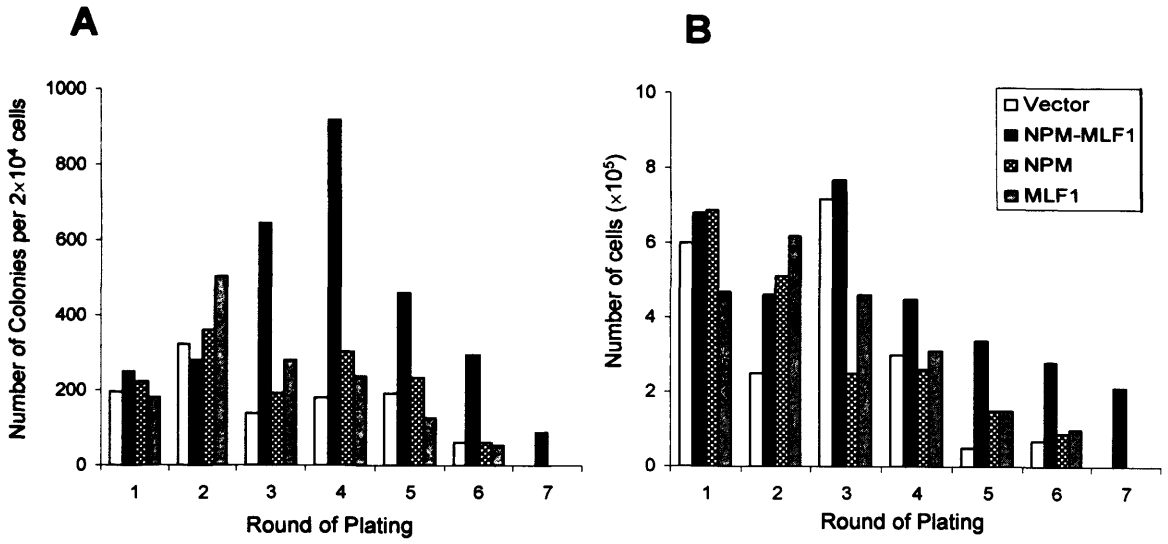


Figure 5.4. HPCs derived from E12.5 FL: NPM-MLF1 enhances self renewal potential of HPCs under myeloid conditions. A. Bar chart showing number of colonies formed at each round of plating. **B.** Bar chart showing number of cells at each round of plating. HPCs isolated from E12.5 FL were transduced with vector, NPM-MLF1, NPM or MLF1 retrovirus. Transduced cells were cultured in 3434 methylcellulose media in the presence of GM-CSF and incubated for 7 days at 37°C.

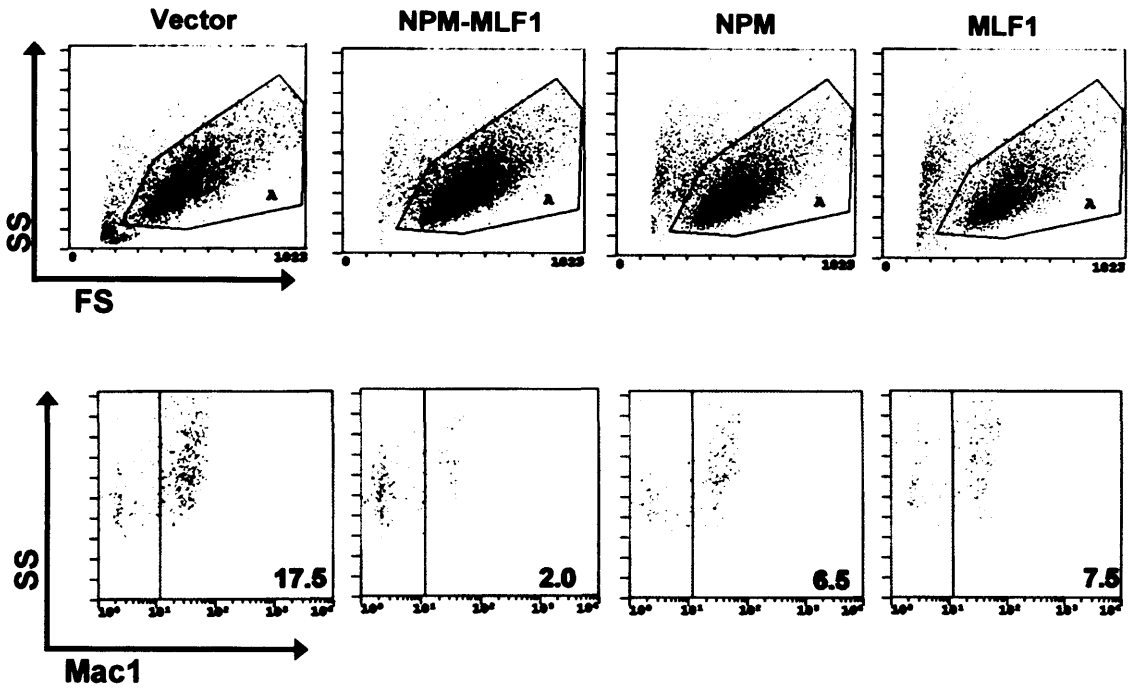


Figure 5.5. HPCs derived from E12.5 FL: NPM-MLF1 inhibits myeloid differentiation. Representative flow cytometry data from the first round of an infection experiment. Dot plot shows the percentage of Mac1 positive cells on HPCs transduced with the indicated retrovirus. The numbers in the plot represent the percentage of positive cells.

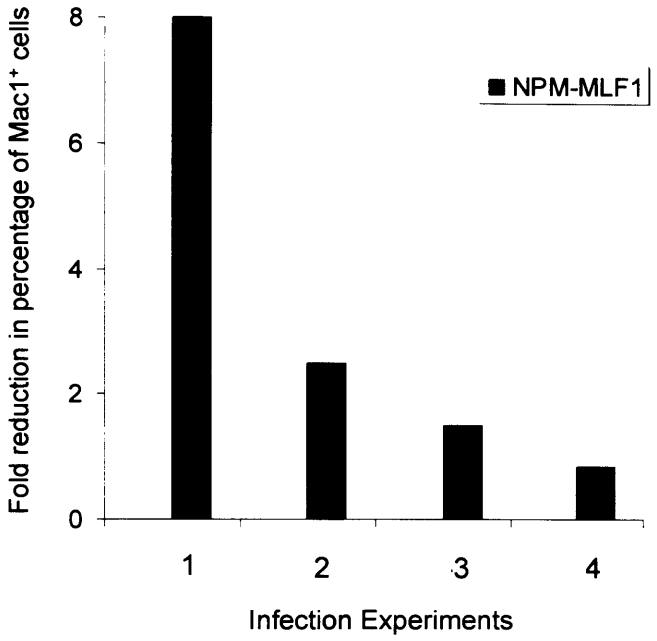


Figure 5.6: Mac1 expression level is reduced on NPM-MLF1 transduced cells. Bar chart showing the fold reduction in the percentage of Mac1 positive cells as compared to vector. HPCs isolated from E12.5 FL were cultured in methylcellulose in the presence of GM-CSF, SCF, IL-3 and L-6 and the transduced cells were selected in G418. Cells were analysed from the first round of plating of four separate infection experiments.

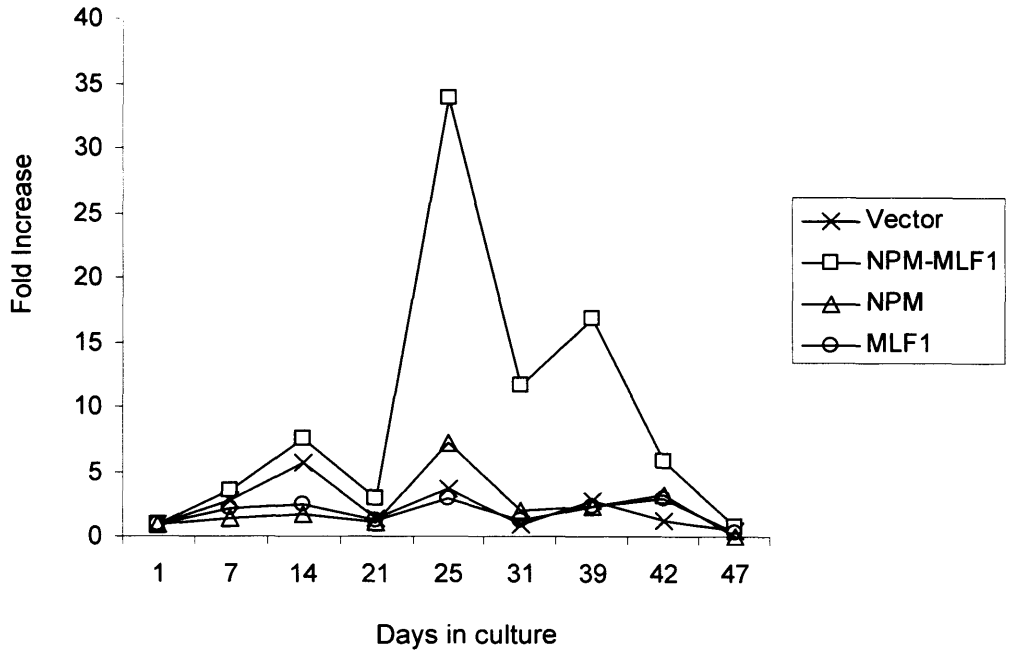


Figure 5.7. Cells transduced with NPM-MLF1 can not be maintained in liquid culture. Fold increase in cell number of cells transduced with vector, NPM-MLF1, NPM or MLF retrovirus. Total cells from the third round of plating were grown in liquid culture supplemented with SCF, IL-3, IL-6 and GM-CSF.

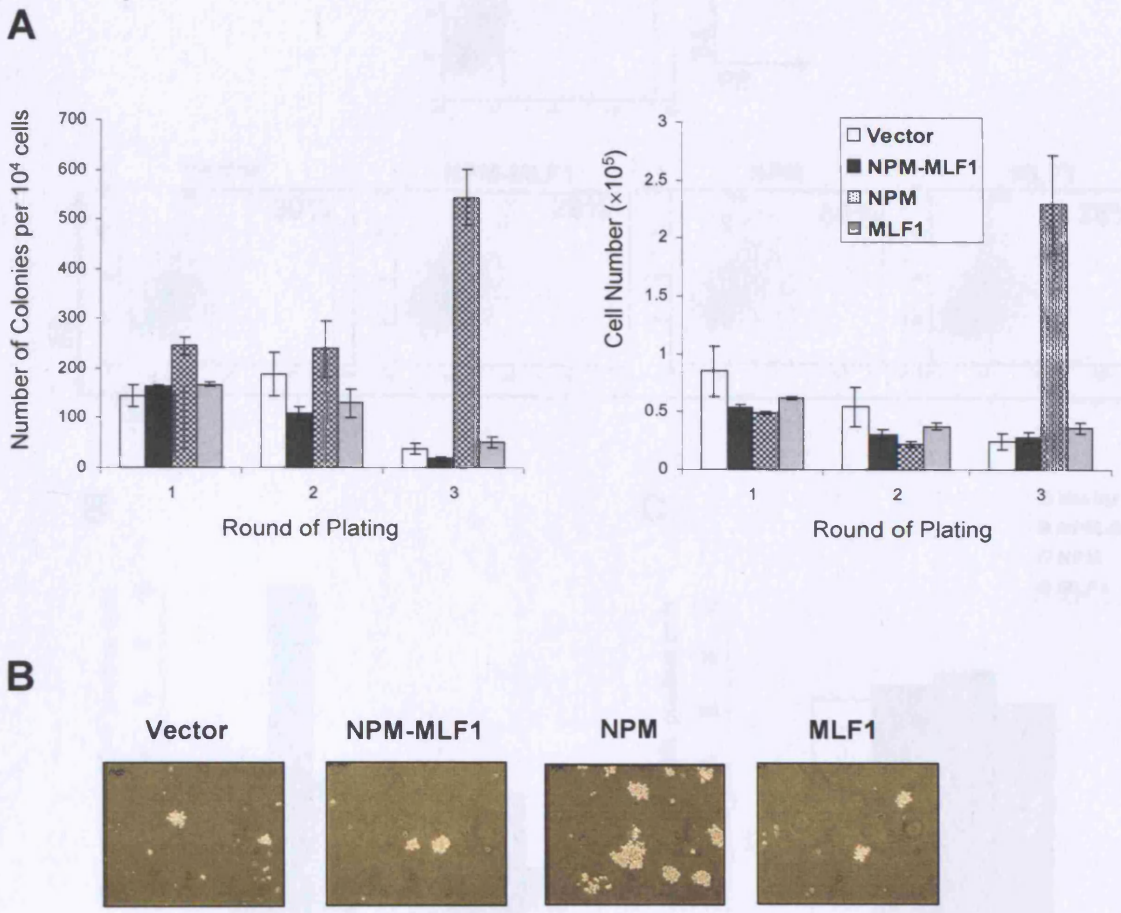


Figure 5.8. NPM enhances colony formation of HPCs under megakaryocytic condition. **A.** Total number of cells (Left panel) and total number of colonies formed was counted and scored at each round of plating. The data represents mean and ± S.D of one experiment performed in duplicate. **B.** Typical morphology of colonies formed by the indicated retrovirus at the third round of plating (original magnification ×100). Progenitor cells were isolated from E12.5 FL and cells were transduced with empty vector, *NPM-MLF1*, *NPM* or *MLF1* retrovirus. The transduced cells were subsequently cultured in methylcellulose 3234 in the presence of TPO and SCF. Cells were grown in culture for 10 days at each round.

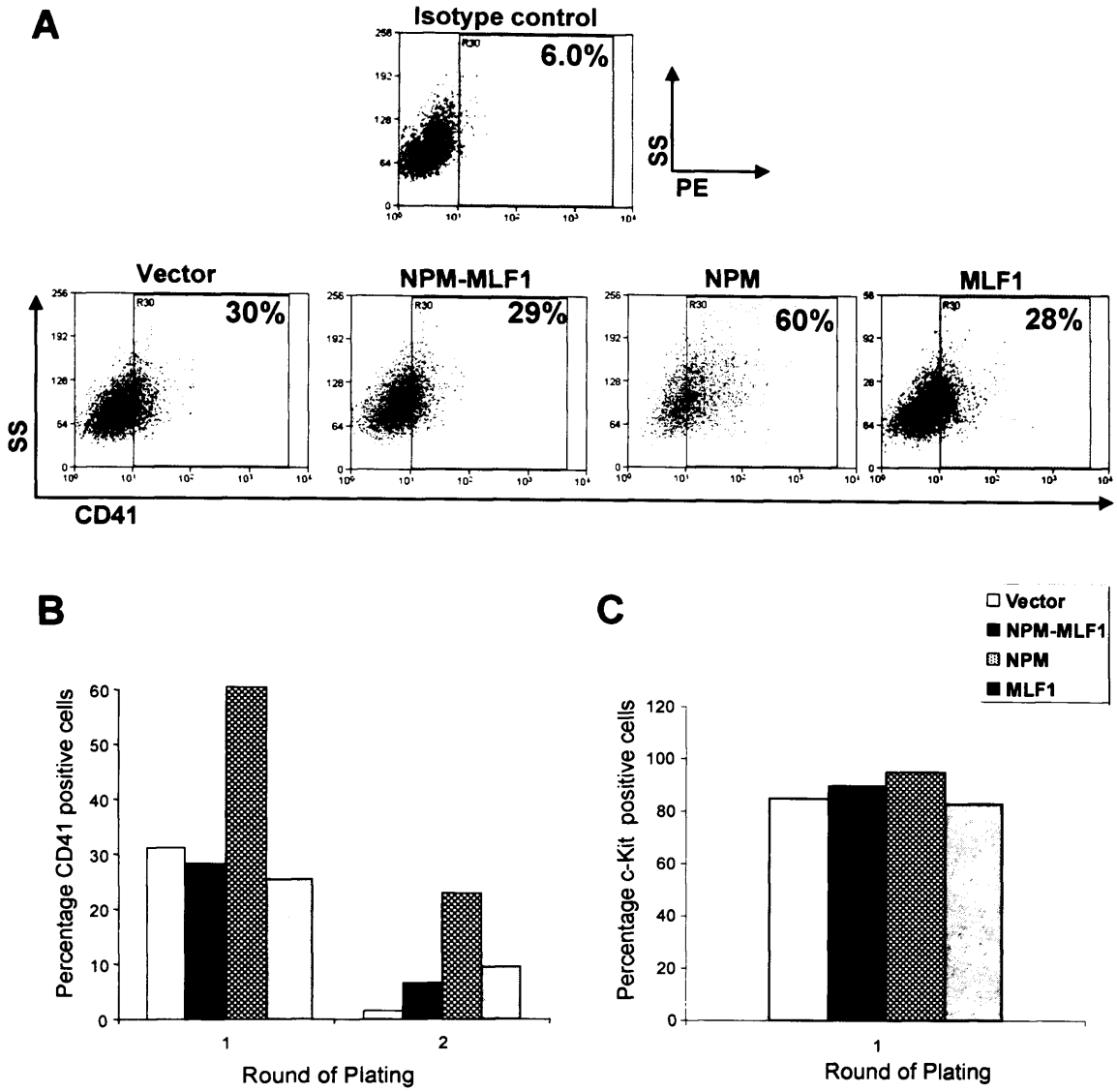


Figure 5.9. CD41 expression level is increased on NPM transduced cells. A. Dot plots represent the percentage of CD41 and c-Kit positive cells from the first round of plating. The number in the plots represent the percentage of positive cells. Flow cytometry performed on cells harvested from first round of plating. **B.** Bar chart showing the percentage of CD41 positive cells from the first and second round of plating. **C.** Bar chart representing the percentage of c-Kit positive cells from the first round of plating. HPCs were isolated from E12.5 FL and were transduced with indicated retroviruses. Cells were plated on methylcellulose media, 3234 supplemented with TPO and SCF.

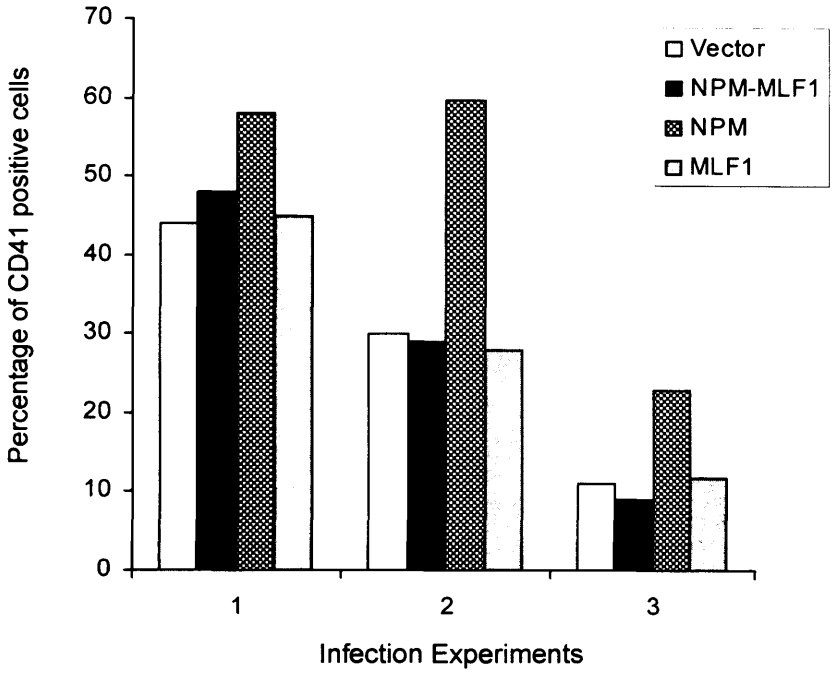


Figure 5.10. NPM promotes megakaryocytic differentiation. Summary of the percentage CD41 positive cells at the first round of plating from three independent infection experiments. There is an increase in CD41 positive cells with cells transduced with NPM as compared with vector, NPM-MLF1 and MLF1 expressing cells.

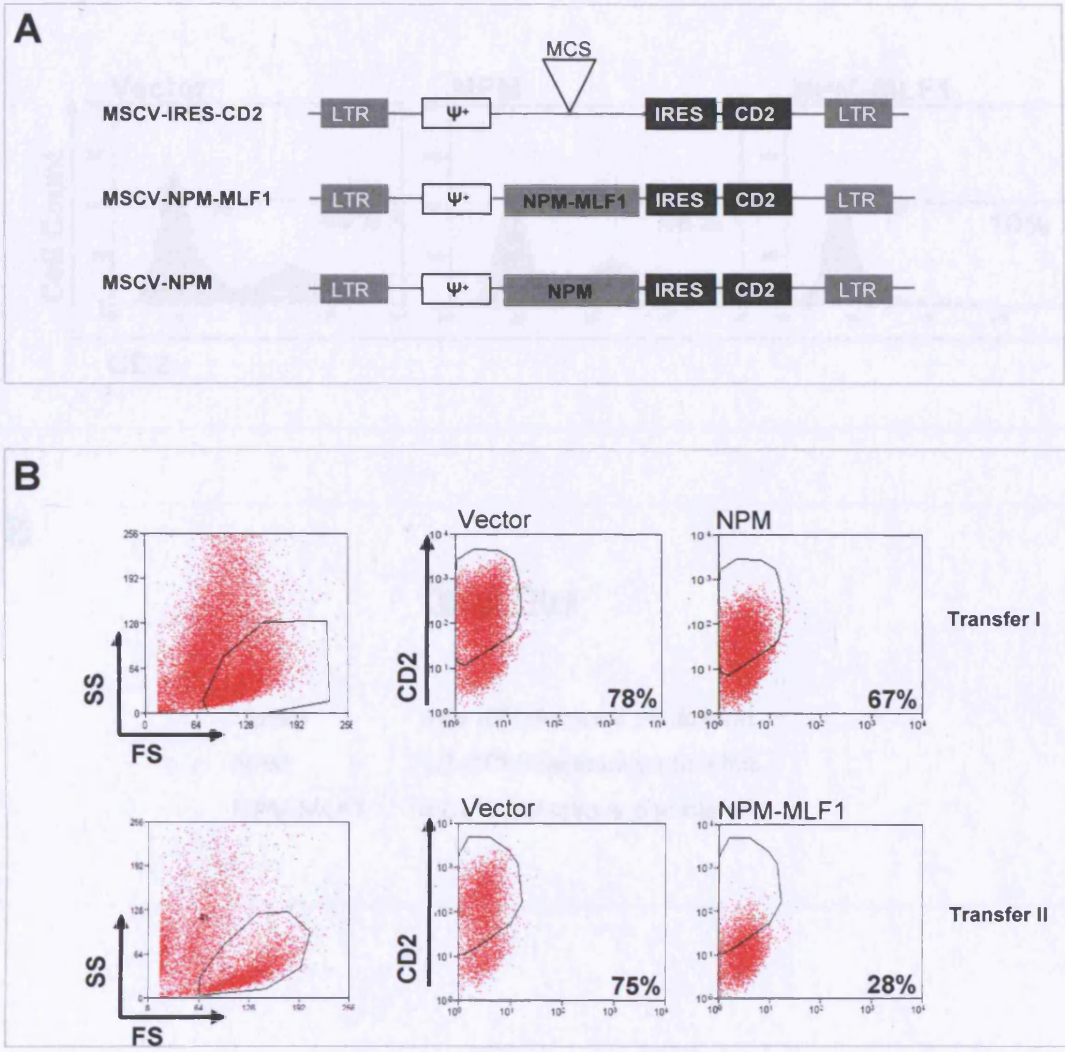
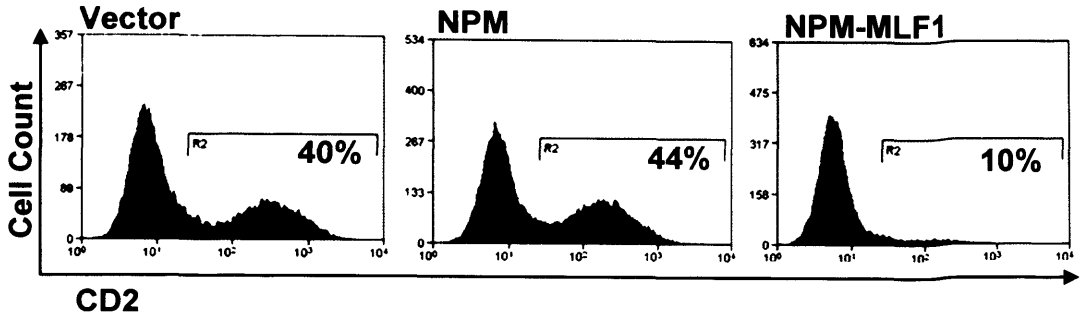


Figure 5.11. Infection efficiency of transduced cells prior to injection into sub-lethally irradiated mice. A. Schematic diagram of pMSCV-IRES-CD2 constructs. *NPM* and *NPM-MLF1* cDNA were cloned into pMSCV retroviral vector. **B.** HPCs were isolated from E12.5 FL and were transduced with either vector alone, *NPM* or *NPM-MLF1* retrovirus. Cells were cultured in myeloid conditions (SCF, IL-3 and IL-6). Infection efficiencies were measured 24 hrs following transduction by measuring the expression of hCD2. Cells were subsequently intravenously injected into sub-lethally γ-irradiated mice. The numbers represent the percentage of positive cells.

A



B

Viral Titre

Vector	1.6×10^6 Infectious particle/mL
NPM	1.7×10^6 Infectious particle/mL
NPM-MLF1	4.0×10^4 Infectious particle/mL

Figure 5.12. NPM-MLF1-IRES-CD2 has a low viral titre. Determination of viral titre for pMSCV-IRES-CD2 retroviruses. Retroviral supernatant was harvested from LinXE cells and diluted at 1:20, 1:200 and 1:2000 with DMEM complete medium. Mouse 3T3 fibroblasts were transduced with either empty vector, *NPM* or *NPM-MLF1* retroviral supernatant at the different dilution. **A.** Histogram showing the expression of hCD2 expression 48 hrs after infection (Retroviral supernatant at 1:200 dilution). **B.** The calculated viral titre for the indicated viruses.

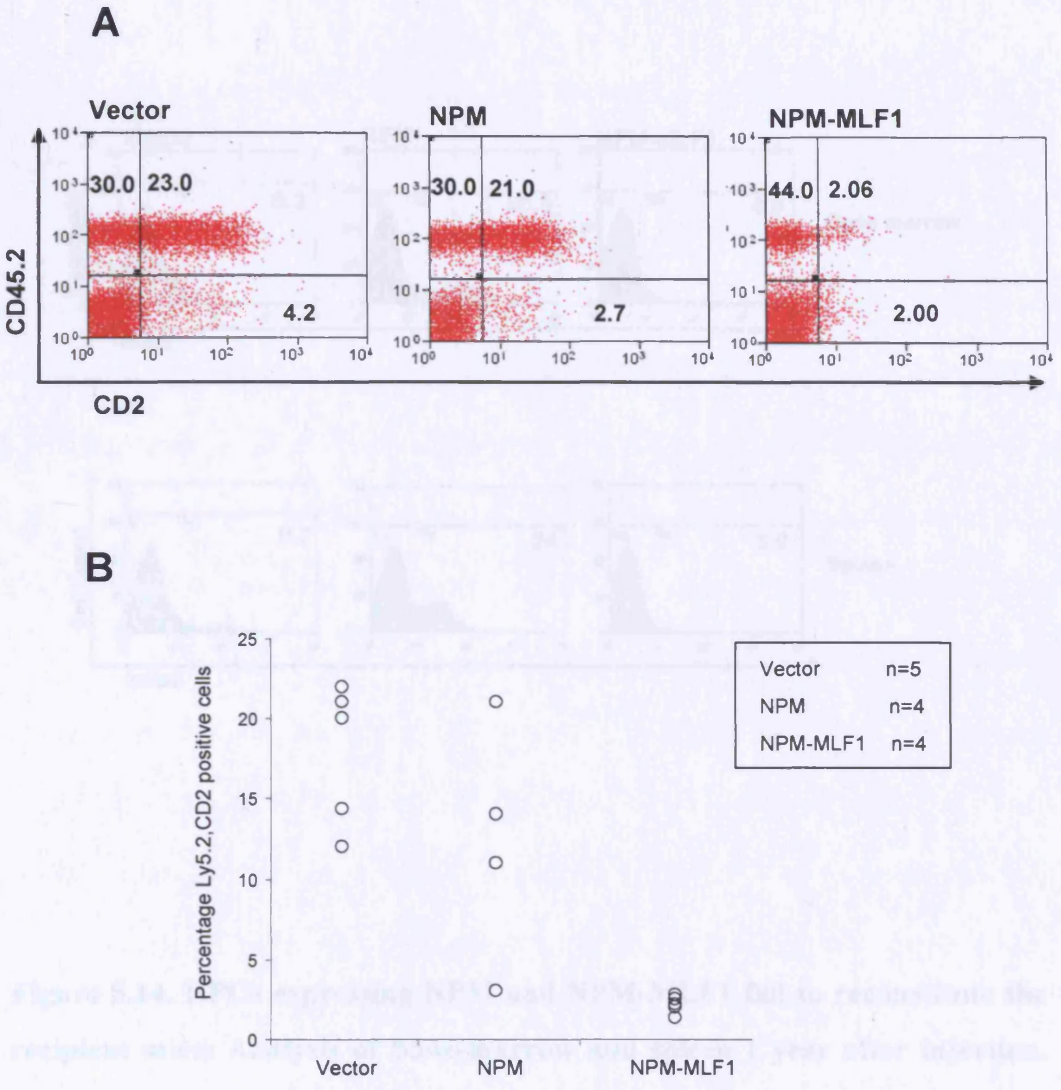


Figure 5.13. HPCs expressing NPM-MLF1 are incapable of short term reconstitution. Analysis of peripheral blood 6 weeks following injection. **A.** Representative dot plots showing the percentage of CD45.2 and CD2 positive cells from mice injected with HPCs expressing vector, NPM or NPM-MLF1. Number represent the percentage of positive cells. **B.** The percentage of CD45.2 and CD2 positive cells injected mice.

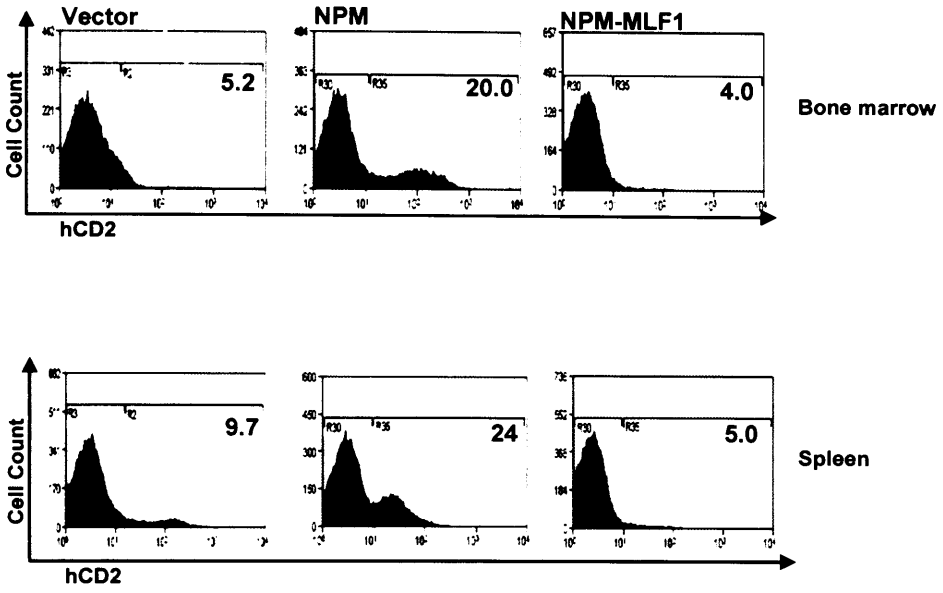


Figure 5.14. HPCs expressing NPM and NPM-MLF1 fail to reconstitute the recipient mice: Analysis of bone marrow and spleen 1 year after injection. Bone marrow and splenocytes were analysed by flow cytometry 1 year and 2 months after injection. Representative histogram showing the percentage of CD2 positive cells from bone marrow (Top panel) and spleen (Bottom panel).

5.5 Discussion

5.5.1 NPM-MLF1 impairs myeloid differentiation

It has been postulated that myelodysplasia detected in MDS occurs as a result of ineffective haematopoiesis and differentiation. The disease is characterised by abnormal proliferation of cells in the bone marrow and high levels of apoptosis in the blood. In MDS, the balanced t(3;5) translocation rearranges MLF1 on chromosome 3 and NPM located on chromosome 5. This translocation leads to the expression of NPM-MLF1 fusion transcript. The contribution of this fusion protein to the pathogenesis of MDS is unknown. Lymphoid cells are not involved in most MDS patients and unlike the mature myeloid cells, are not derived from the MDS clone. It is therefore predicted that the initial transformation event may occur in a myeloid restricted progenitor cell. However, it is highly likely that the primary genetic changes in MDS occur at the HSC level, and the effect of these transformation events is myeloid restricted, leaving the commitment and differentiation of lymphoid progenitor unaffected. It is likely that the B and T lymphocytes in MDS patients are derived from the residual normal HSCs. The cells that define the disease are clonally derived and since trilineage dysplasia in MDS affects cells of the myeloid and megakaryocytic lineage, we set out to investigate the growth and differentiation effects of NPM-MLF1 in these lineages.

Based on colony growth assays, we have shown that NPM-MLF1 impairs myeloid differentiation, as characterised by the reduction in the levels of Mac1 and Gr1 expression. This is coupled to an increase in the proportion of immature c-Kit positive progenitor cells, which are the only cells detected in the NPM-MLF1

cultures in the first round of plating. The absence of differentiated cells in the subsequent rounds further suggests that NPM-MLF1 blocks differentiation. The effect of NPM-MLF1 can be seen in both foetal and adult progenitor cells. One explanation for these results is that NPM-MLF1 negatively regulates genes required for differentiation of progenitor cells. Alternatively, the accumulation of c-Kit positive progenitor suggests that NPM-MLF1 may increase self renewal potential or enhance proliferation of these cells.

Cells transduced with NPM-MLF1 also have a transient increase in their capacity to form myeloid colonies. Colonies formed by the NPM-MLF1 expressing cells were of a smaller and more compact size and probably represent a less differentiated progenitor population, as compared to control cells. However, the fusion protein does not immortalise the transduced progenitor cells in colony growth assays or in liquid culture. A secondary event may be required for the immortalisation of these cells. Inhibition of apoptosis is a central component to the progression and evolution of MDS to AML. This is supported by the studies showing increased expression of the pro-apoptotic (BAX/BAD) versus anti-apoptotic (BCL2/BCLXL) proteins in the CD34 compartment of patients with MDS (Parker *et al.*, 1998). A decrease in this ratio is associated with evolution to leukaemia (Parker et al., 1998; Boudard *et al.*, 2002). Similar alterations in this ratio have been associated with expression of fusion proteins. For example, BCR/ABL expression in myeloid progenitor cells was shown to promote myeloproliferative disorder whereas its co-expression with BCL2 lead to AML development in mice (Jaiswal *et al.*, 2003). *In vitro* studies in NIH3T3 cells have shown that NPM-MLF1 induced apoptosis upon serum starvation. Control NIH3T3 cells and cells transfected with BCL2 alone failed to

proliferate under these conditions (Yoneda-Kato *et al.*, 1999). Interestingly however, BCL2 rescued NPM-MLF1 mediated apoptosis and more than 90% of cells incorporated BrdU under these conditions, thus suggesting that expression of both BCL2 and NPM-MLF1 confer a growth advantage to cells. It is therefore possible that NPM-MLF1 may collaborate with BCL2 or BCLXL to immortalise HPCs in a sub-optimal environment.

The mechanism underlying the multi-step pathogenesis of MDS is as yet unclear. The peripheral cytopenia occurs as a result of apoptosis that is detected in the early stages of the disease. The apoptosis can be partially explained by the deregulation of BCL2 proteins and other apoptotic pathways. A general increase in apoptosis in haematopoietic cells would be predicted to result in hypocellular BM. It may be possible that in MDS patients, NPM-MLF1 increases the accumulation of immature progenitor cells and blocks their differentiation (**Figure 5.15**). The accumulation of cells results in a hypercellular BM which is a characteristic phenotype of MDS. The increased apoptosis may be due to ineffective or aberrant differentiation and maybe a mechanism to prevent acquisition of further mutations culminating in leukaemic transformation. Expression of the fusion protein is probably not sufficient to transform a cell but it may instigate a so called "pre-leukaemic" state. As the disease progresses, clonal progenitor cells expressing NPM-MLF1 accumulate genetic events that would allow the cell to overcome the pro-apoptotic signals. Acquisition of additional mutations that would cooperate with the fusion protein to enhance the survival or proliferative advantage of a cell could ultimately result in the progression of MDS to AML.

The impaired differentiation and the transient increase in colony formation detected by NPM-MLF1 expressing cells recapitulate some of the clinical features that are detected in MDS patients. Our model can thus be very useful *in vitro* to further analyse the mechanism in which NPM-MLF1 contributes to MDS pathogenesis.

5.5.2 NPM-MLF1 does not affect megakaryopoiesis

The ineffective megakaryopoiesis detected in MDS patients is reflected in the peripheral thrombocytopenia and dysplasia in megakaryocytes. Dysplasia is characterised by abnormalities in cell size, failure of nuclear segmentation in megakaryoblasts and nuclear hypersegmentation in mature megakaryocytes. MDS patients with the NPM-MLF1 fusion have megakaryodysplasia but there is no direct evidence implicating the involvement of the fusion protein in the aberrant maturation of these cells. Therefore, we set out to investigate the role of NPM-MLF1 in megakaryocytic development.

Foetal liver progenitor cells were transduced with *NPM-MLF1* retrovirus. By performing colony-forming assays under conditions that promote megakaryopoiesis, we were able to examine the effect of NPM-MLF1 protein on the development of these cells. Our data suggests that NPM-MLF1 does not have any effect on megakaryopoiesis. There was no difference in the percentage of CD41 positive cells or in number of colonies formed by the NPM-MLF expressing cells as compared to vector control cells. This implies that the megakaryodysplasia detected in MDS patients is not directly related to the NPM-MLF1 chimeric protein. Although no other chromosomal abnormalities were detected in these patients, it is conceivable that other genetic alterations exist in genes that are essential for regulating

megakaryocytic development. The responsiveness of progenitor cells to TPO, abnormal expression of TPO receptor or defect in signal transduction pathways can also explain the ineffective maturation of megakaryocytes detected in MDS. Alternatively, the effect of NPM-MLF1 may only be evident in BM-derived progenitor cells. For future studies, colony forming assays should be carried out using lineage negative BM cells transduced with NPM-MLF1. In addition, there may be a difference between the activities of NPM-MLF1 in murine versus human progenitors.

However, our preliminary data suggest that regulation of NPM may be essential in megakaryocytic development. *In vitro* colony assays show that progenitor cells transduced with NPM have an increased megakaryocytic differentiation potential as measured by the expression of CD41 There was also an increase in the clonogenic potential of NPM expressing cells. In common with our results, ectopic expression of NPM in LSK (Lin^-, $Sca1^+$, $c\text{-}Kit^+$) cells results in increased proliferation of these cells (Li *et al.*, 2006). It is possible that in MDS, NPM-MLF1 may have a dominant negative effect on endogenous NPM and as a result suppress megakaryocytic differentiation. Alternatively, NPM gene dosage may be an important factor in the regulation of megakaryopoiesis. Therefore, the loss of one wild type *NPM* allele as a result of chromosomal translocation in MDS may have an inhibitory effect on megakaryocytic development. In accordance with this, loss of an *NPM* allele in $NPM^{+/-}$ mice resulted in many features observed in human MDS, such as dysplastic megakaryocytes, (Grisendi et al., 2005). Furthermore, these mice had a higher propensity for developing haematological malignancies as compared with *NPM* wild type mice (Sportoletti et al., 2008), suggesting that quantative abnormality of

NPM may be a crucial contributing factor in the pathogenesis of MDS and progression to AML.

5.5.2 Transplantation of NPM-MLF1 expressing cells

The capacity of NPM-MLF1 to affect myeloid differentiation and transiently enhance cell survival indicates that our model system recapitulates some of the biological properties of human MDS *in vitro*. This led us to examine the effect of fusion expression *in vivo*. Cells transduced with NPM-MLF1, NPM or vector, were injected into sub-lethally irradiated mice. This system allowed us to investigate how efficiently NPM-MLF1 expressing cells repopulate the mouse haematopoietic system and what effect the fusion had on the development of the myeloid lineage.

Transduction efficiency, as measured by hCD2 expression was approximately 70% and 60% for cells expressing vector and NPM, respectively. In contrast, the hCD2 expression of NPM-MLF1 expressing cells was as low as 30%. The difference in the levels of hCD2 expression was shown to be due to the production of low viral titre from the NPM-MLF1 retroviral construct. To determine the level of short term reconstitution in these mice, the peripheral blood of recipient mice was analysed 6 weeks post injection. The inability to detect donor cells expressing NPM-MLF1 is probably due to the low infection efficiency of transplanted cells. Since the retroviral supernatant used to infect progenitor cells was already concentrated, we could not further increase the viral titre produced by this construct. The presence of an IRES can interfere with the expression levels of CD2 detected in cells. IRES mediated translation is dependent on the interaction of cellular proteins with RNA in the translation initiation complex, a process that can be regulated by cell type or

cell status (Borman *et al.*, 1995; Stoneley *et al.*, 2000). Indeed, the level of CD2 expression is much higher in 3T3 cells or LinXE cells than when murine progenitor cells are transduced with *NPM-MLF1* retrovirus. For future studies, *NPM-MLF1* should be cloned into a retroviral expression vector lacking IRES. Alternatively, since hCD2 expression was not affected with the empty vector, the NPM-MLF1 cDNA may interfere with the function of an IRES and hence the expression of hCD2, which could result in miss-representation of transduced cells.

In contrast to the data presented by Pang's group, cells overexpressing NPM did not affect the myeloid lineage in our system. Li *et al* showed that NPM expressing progenitors from bone marrow of 5-FU treated mice had increased reconstitution in syngeneic mice. NPM expressing cells had reduced propensity to commit to the myeloid lineage and in contrast to our study, NPM inhibited myeloid differentiation. Secondary transplantation also revealed the long term repopulating capability of HSCs expressing NPM (Li et al., 2006). Differences in the progenitor cell targeted by NPM could account for the lack of reconstitution observed in our system. The current data on the role of NPM in haematopoiesis is based on colony forming assay using transduced bone marrow derived progenitor cells or the injection of these cells into recipient mice. It is possible that NPM has distinct functions in foetal and adult haematopoiesis. This can be addressed by transducing BM derived progenitor cells and their subsequent injection into irradiated recipient mice to determine the effect of NPM-MLF1 in haematopoiesis and transformation. Another source of BM derived progenitor cells could be obtained from the $NPM^{+/-}$ mice, which have been obtained from Grisendi *et al* (Grisendi et al., 2005). The progenitor cells from these mice would be ideal in analysing the functional contribution of NPM-MLF1 to

MDS as the NPM haploinsufficiency resembles to the progenitor cells in MDS patients.

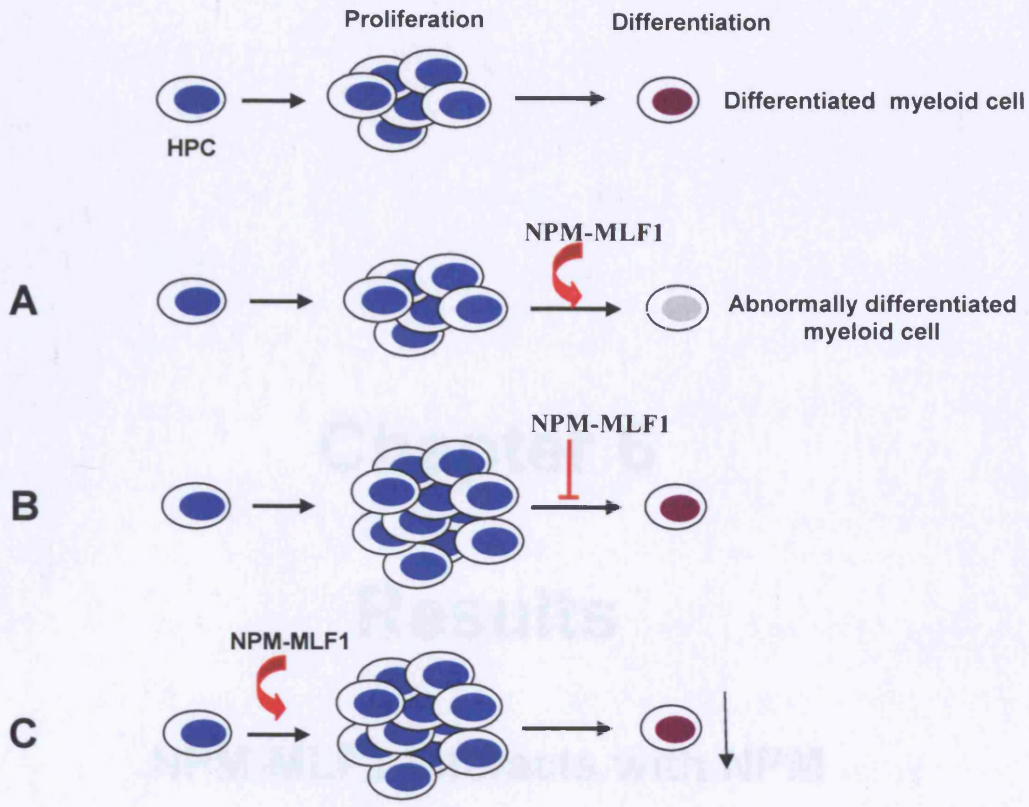


Figure 5.15. Possible roles of NPM-MLF1 in myelopoiesis. There are number of factors that can regulate different stages of HPC development. HPCs can proliferate and commit to differentiate along a specific lineage. NPM-MLF1 fusion protein may act at distinct stages of myeloid development. **A.** In MDS, NPM-MLF1 may fail to differentiate effectively. These cells can appropriately proceed along a specific pathway but are unable to terminally differentiate, resulting in production of abnormally differentiated cells. **B.** Alternatively, NPM-MLF1 may block differentiation, causing accumulation of immature progenitor cells. **C.** NPM-MLF1 could increase HPC self-renewal at the expense of differentiation.

Chapter 6

Results

NPM-MLF1 interacts with NPM

Chapter 6: NPM-MLF1 interacts with NPM

NPM is involved in a number of translocations that are detected in individuals with different leukaemic subtypes. In the t(5;17) translocation *NPM* is fused with the retinoic acid receptor-α (*RAR-α*) gene and the resultant fusion gene is associated with the pathogenesis of acute promyelocytic leukaemia (APL) (Redner et al., 1996). In anaplastic large cell lymphoma (ALCL), *NPM* is fused to the anaplastic lymphoma kinase receptor gene (*ALK*). The chimeric protein generated results in the constitutive activation of ALK tyrosine kinase function (Morris et al., 1994). NPM-ALK has been shown to interact with endogenous NPM (Bischof *et al.*, 1997). This association is important for the localisation of NPM-ALK to the nucleus, and is essential for transformation of cells by the fusion protein. The identification of proteins that interact with NPM-MLF1 will allow a better understanding of the function of this fusion protein. As the homodimerization domain is retained in all NPM fusion proteins, we set out to investigate if NPM-MLF1 interacts with NPM and to determine if this interaction has any functional significance.

6.1 NPM-MLF1 interacts with NPM

To determine whether NPM-MLF1 interacts with NPM, immunoprecipitations were performed using whole cell lysates from mouse 3T3 fibroblasts. These cells were transduced with *NPM-MLF1* retrovirus. The transduced cells were selected by growth in G418. To determine whether NPM interacts with NPM-MLF1, whole cell lysates were prepared from the transduced 3T3 cells, using RIPA lysis buffer. The lysates were pre-cleared with protein A coupled agarose beads for 1 hr at 4°C. Initially, immunoprecipitation was carried out using a mouse NPM monoclonal

antibody. The antibody was raised against the C-terminal portion of NPM that is truncated in the fusion protein and is replaced with MLF1. The use of this antibody enabled us to differentiate between the endogenous NPM and NPM-MLF1.

The precipitated protein complexes were analysed on SDS-PAGE. The fusion protein was tagged with a FLAG epitope at the N-terminus and a FLAG antibody was used to detect the presence of co-precipitated NPM-MLF1 fusion protein (**Figure 6.1A**). In order to measure the relative expression of the protein that is precipitated, 10% of the whole cell lysate was used to run on the gel. Lysates from untransduced 3T3 cells and cells transduced with the vector alone were used as a control (lanes 1 and 2 respectively). A protein of approximately 48 kDa was detected in the whole cell lysate (lane 3), corresponding to the FLAG tagged fusion protein. The FLAG-NPM-MLF1 protein was detected in the precipitated NPM complex (lane 6) and not in control cell lysates. This indicates that in 3T3 cells, endogenous NPM interacts with NPM-MLF1.

In order to further confirm the interaction between NPM and NPM-MLF1, FLAG antibody was used to precipitate NPM-MLF1 protein and to establish whether the endogenous NPM protein is within the precipitated complex. The interaction of FLAG-NPM-MLF1 with the 38 kDa NPM protein was revealed by immunobloting with an anti-NPM antibody (**Figure 6.1B**). The immunoprecipitates revealed an association between the fusion protein and NPM (lane 6). High levels of NPM protein were present following immunodepletion with the FLAG antibody, suggesting that only a proportion of endogenous NPM interacts with NPM-MLF1 (lanes 10-12). These data show that NPM-MLF1 interacts with endogenous NPM.

6.2 NPM-MLF1 is localised to the nucleoli

Subcellular localisation of NPM-MLF1 in 3T3 cells was determined by immunostaining. Cells were grown on coverslips and fixed with paraformaldehyde. In order to permeabilise the cells, they were treated with Triton X-100. Cells were stained with Hoechst dye which stains chromosomes within the nucleus (**Figure 6.2, right panel**). FLAG antibody was used to detect cells expressing the NPM-MLF1 fusion protein. One cell within the highlighted section is expressing NPM-MLF1 and the merged image shows the localisation of the protein to the nucleoli (**Figure 6.2, merged image**).

6.3 Generation of deletion constructs to determine the domain required for the interaction between NPM-MLF1 and NPM

Several retroviral deletion constructs were generated to determine which domains of NPM-MLF1 contribute to the interaction with NPM. The first deletion construct, truncated NPM (tNPM), comprised the N-terminal domains of NPM retained in the NPM-MLF1 fusion protein. tNPM contains two nuclear export signals (NES), the metal binding domain, the acidic domains and one nuclear localisation signal (NLS) **(Fig 6.3B)**. In theory, tNPM protein could shuttle between the cytoplasm and the nucleus due to the presence of NES and NLS. It has been shown that this segment of NPM is responsible for the localisation in the nucleoli and nucleoplasm (Bertwistle *et al.*, 2004). As NPM is mainly localised in the nucleoli, a construct was generated in which the NLS domain was deleted (NPM-ΔNLS). This construct would allow us to establish the importance of NPM cellular localisation for interaction with NPM-MLF1, since the absence of the NLS would prevent NPM localisation to the nucleus

and hence prevent interaction with endogenous NPM, which is predominantly present in the nucleoli. In addition, NPM shuttling activity between the cytoplasm and nucleus would be affected. Indeed, Bertwistle *et al* have shown the localisation of NPM-ΔNLS protein to be predominantly in the cytoplasm (Bertwistle et al., 2004).

To determine if MLF1 is required for the interaction of the fusion protein with NPM, truncated MLF1 (tMLF1) was also generated. This deletion mutant corresponded to the MLF1 moiety present in NPM-MLF1. It has been reported that MLF1 is sequestered in the cytoplasm by the 14-3-3 protein. Since NPM-MLF1 is present in the nucleoli, an NPM-MLF1 construct was generated containing only the NPM derived NLS joined to the MLF1 moiety (MLF1-NLS). The presence of the NPM-NLS should be sufficient to allow the translocation of MLF1 into the nucleus, where NPM is located. Co-immunoprecipitation would reveal if the MLF1 portion of the fusion protein is required for the interaction with NPM. The expression of proteins was determined in transduced 3T3 cells. Western blot analysis of total cell lysates revealed the expression of Flag tagged proteins of the expected molecular weights **(Figure 6.4).**

6.4 The NPM moiety of NPM-MLF1 is required for interaction with NPM

The deletion mutants were retrovirally expressed in mouse 3T3 fibroblasts. The cells were selected in G418 for 10 days and total cell lysates were used in co-immunoprecipitations. FLAG tagged NPMt was shown to interact with endogenous NPM. It is likely that the oligomerization domain at the N-terminal portion of NPM

mediates this interaction. The NPM mutant lacking the NLS domain (NPM-ΔNLS) was also co-immunoprecipitated with NPM **(Figure 6.5)**. Although NPM is primarily found in the nuclei, reports have shown that it can also shuttle between the nucleus and the cytoplasm. It is therefore possible that NPM-ΔNLS, which is localised to the cytoplasm, interacts and forms a complex with the residual endogenous NPM in the cytoplasm. FLAG tagged MLF1t or MLF1-NLS proteins did not co-immunoprecipitate with NPM **(Figure 6.5)**. These data show that the NPM segment of the NPM-MLF1, and not MLF1, is required for interaction with NPM.

6.5 NPM activates p53 reporter activity

NPM interacts with many proteins, such as HDM2, ARF and p53 (Colombo et al., 2002; Kurki et al., 2004; Colombo *et al.*, 2005; Lambert and Buckle, 2006). NPM expression has been shown to regulate p53 protein stability and transcriptional activity (Colombo et al., 2002; Maiguel *et al.*, 2004). The exact effect on the p53 function appears to be dependent on the level of NPM present within a cell. To examine the functional significance of NPM-MLF1 interaction with NPM, the effect of NPM-MLF1 expression on the p53 luciferase reporter activity was examined.

Mouse 3T3 cells were tranfected with vector alone, NPM-MLF1 or NPM expression vectors. Cells were co-transfected with a p53 luciferase promoter comprising of 13 repeats of a p53 consensus sequence (el-Deiry et al., 1992) and with an expression vector encoding the p53 protein. As a control for p53 activation of the p53 promoter, cells were also transfected with a mutant p53 promoter

comprising of 15 repeats of a similar sequence with mutations at critical positions (el-Deiry et al., 1992) **(Figure 6.6A)**. Cells were also co-transfected with a renilla luciferase reporter gene to normalise for transfection efficiency. Cells were harvested, and p53 luciferase activity was measured, 24 hr after transfection. The data in **Figure 6.6B** show the fold change in the p53 luciferase activity. The luciferase activity measured from cells transfected with wild type or mutant p53 promoters, in the absence of p53 expression vector, represent the basal level of p53 activity in this experiment. Co-transfection with the p53 expression vector resulted in the activation of the wild type promoter, but not the mutant p53 promoter. NPM-MLF1 does not enhance or inhibit p53 activation of the luciferase promoter. In contrast to the fusion protein, NPM was shown to significantly enhance p53 activation of the wild type p53 luciferase reporter **(Figure 6.6B)**. These data show that NPM activates p53 promoter activity.

6.6 NPM-MLF1 inhibits NPM mediated activation of p53 promoter

We have shown that NPM-MLF1 interacts with endogenous NPM in mouse 3T3 fibroblasts. NPM-MLF1 may act as a dominant negative protein inhibiting the function of NPM. To address this, we ectopically overexpressed NPM and NPM-MLF1 and examined the effect on the p53 luciferase activity. Co-expression of NPM with NPM-MLF1 resulted in marked inhibition of p53 promoter activation as compared to activation observed when cells were transfected with NPM alone **(Figure 6.7)**. These preliminary results show that NPM-MLF1 may have a dominant negative effect by interacting with NPM and preventing its normal function.

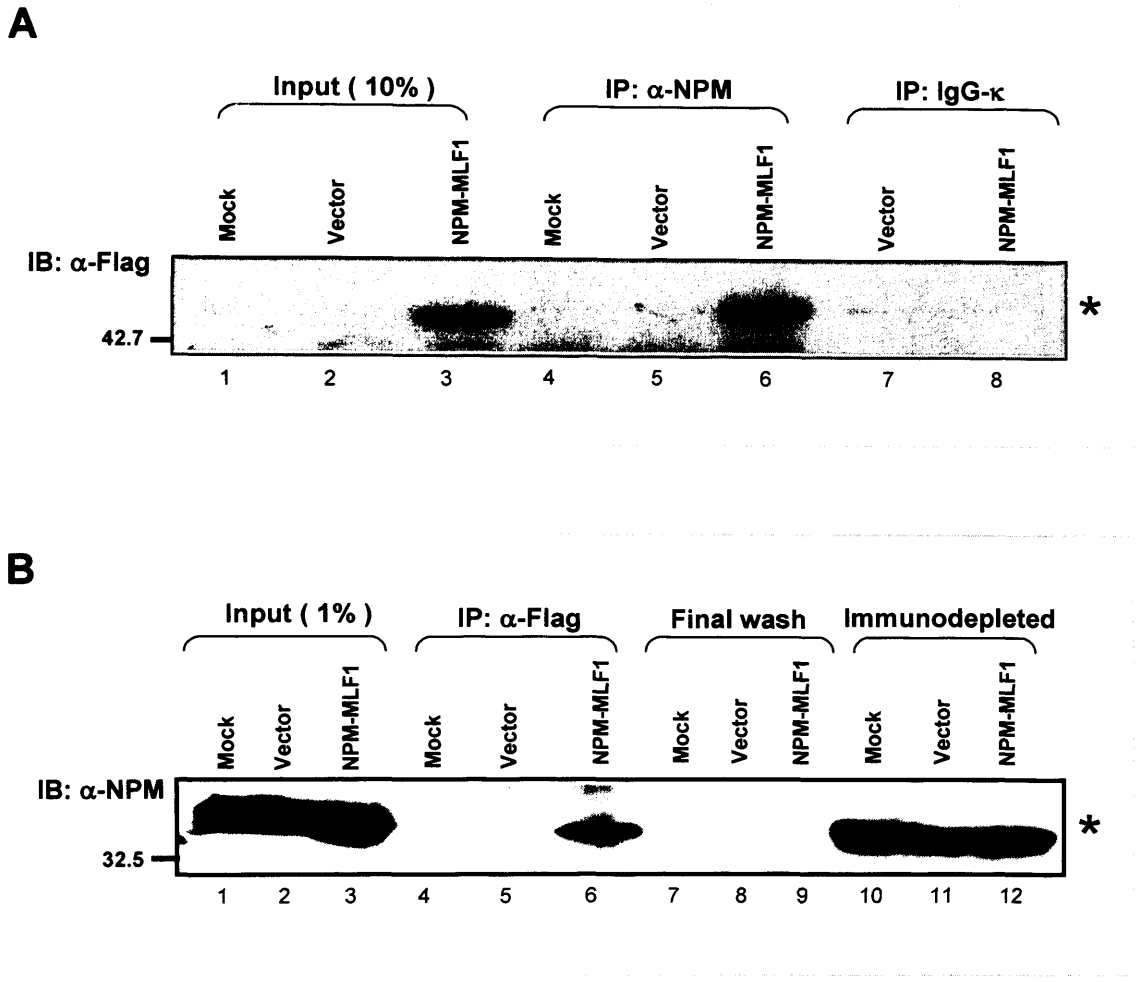


Figure 6.1. NPM-MLF1 interacts with NPM. Mouse 3T3 fibroblasts were transduced with vector alone or *NPM-MLF1* retrovirus. Cell lysates were prepared with RIPA lysis buffer and the immunoprecipitated complexes were resolved on a 10% SDS polyacrylamide gel. **A.** Whole cell lysates were immunoprecipitated with an antibody against NPM or with an IgG-κ antibody as an isotype control and immunobloted (IB) with FLAG antibody. The 48kDa NPM-MLF1 protein (Asterisk) is detected within the immunoprecipitated complex. **B.** Whole cell lysates were immunoprecipitated with a FLAG antibody and immunobloted with an NPM antibody. 40kDa NPM protein (Asterisk) is detected within the immunoprecipitated Flag-NPM-MLF1 complex.

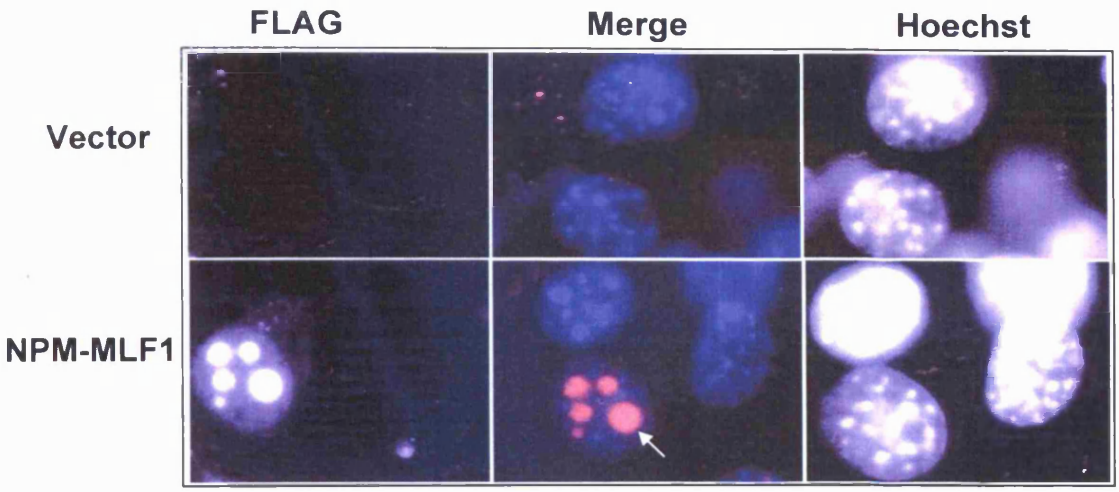


Figure 6.2. NPM-MLF1 is localised in the nucleoli. Transduced 3T3 cells were grown on coverslips. Cells were fixed and permeablised at RT for 5 min. Following several rounds of washing, cell were blocked and labeled with an anti-FLAG antibody. Cells were washed in PBS and incubated with a secondary Rhodamine antibody. The nucleus was stained with Hoechst dye and the localisation of the fusion protein in the nucleoli is shown in the merged image. Cells transduced with empty vector retrovirus were used as a negative control.

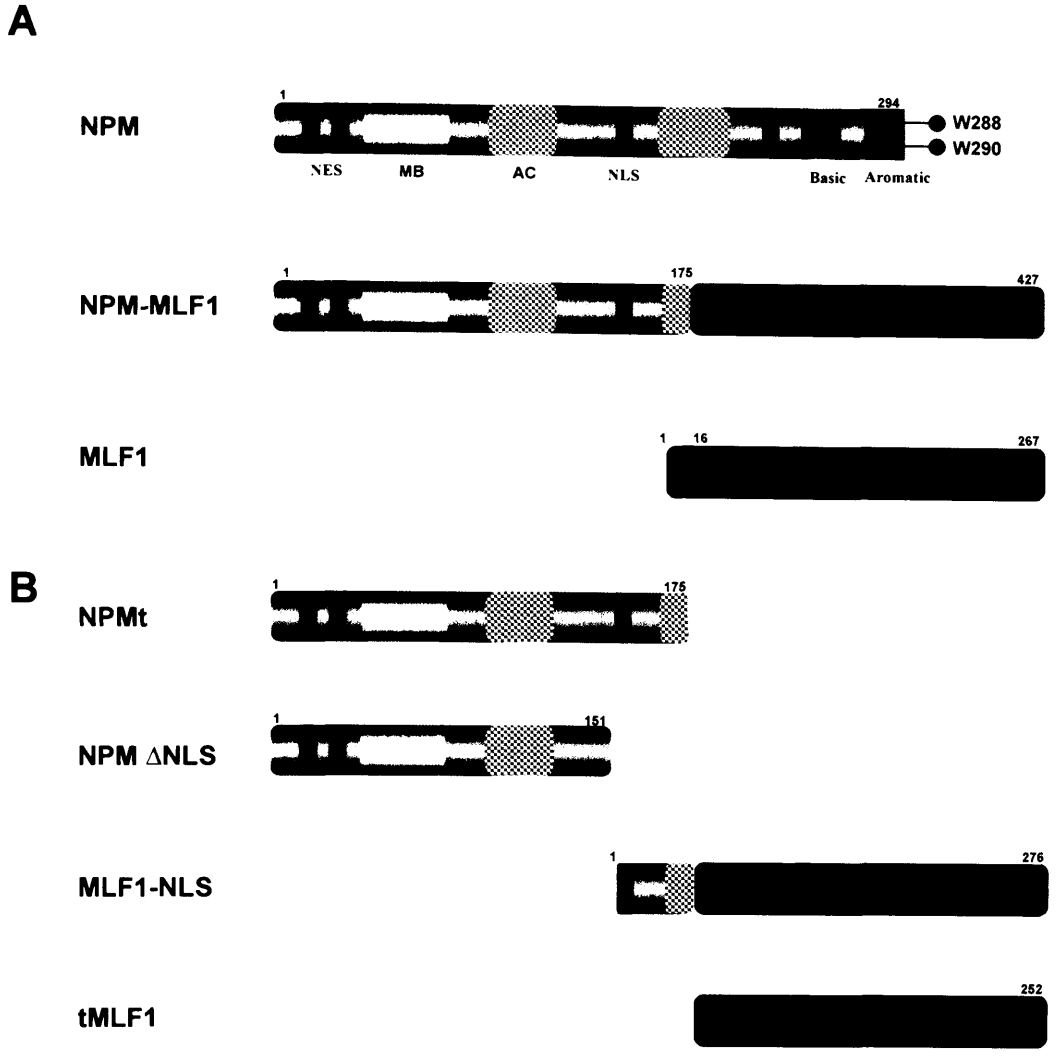


Figure 6.3: NPM and MLF1 deletion constructs. A. Full length wildtype NPM, MLF1 and NPM-MLF1 fusion protein. **B.** Diagram depicting the functional domains in the deletion constructs generated. cDNAs were cloned into pMSCV-neo retroviral vector. Deletion constructs NPMt includes the section of NPM that is retained after t(3;5) translocation. NPM-ΔNLS is truncated NPM lacking the nuclear localisation signal. MLF1-NLS is truncated MLF1 retained in the fusion protein containing a segment of NPM which has the NLS signal. tMLF1 entails the region of MLF1 retained after t(3;5) translocation.

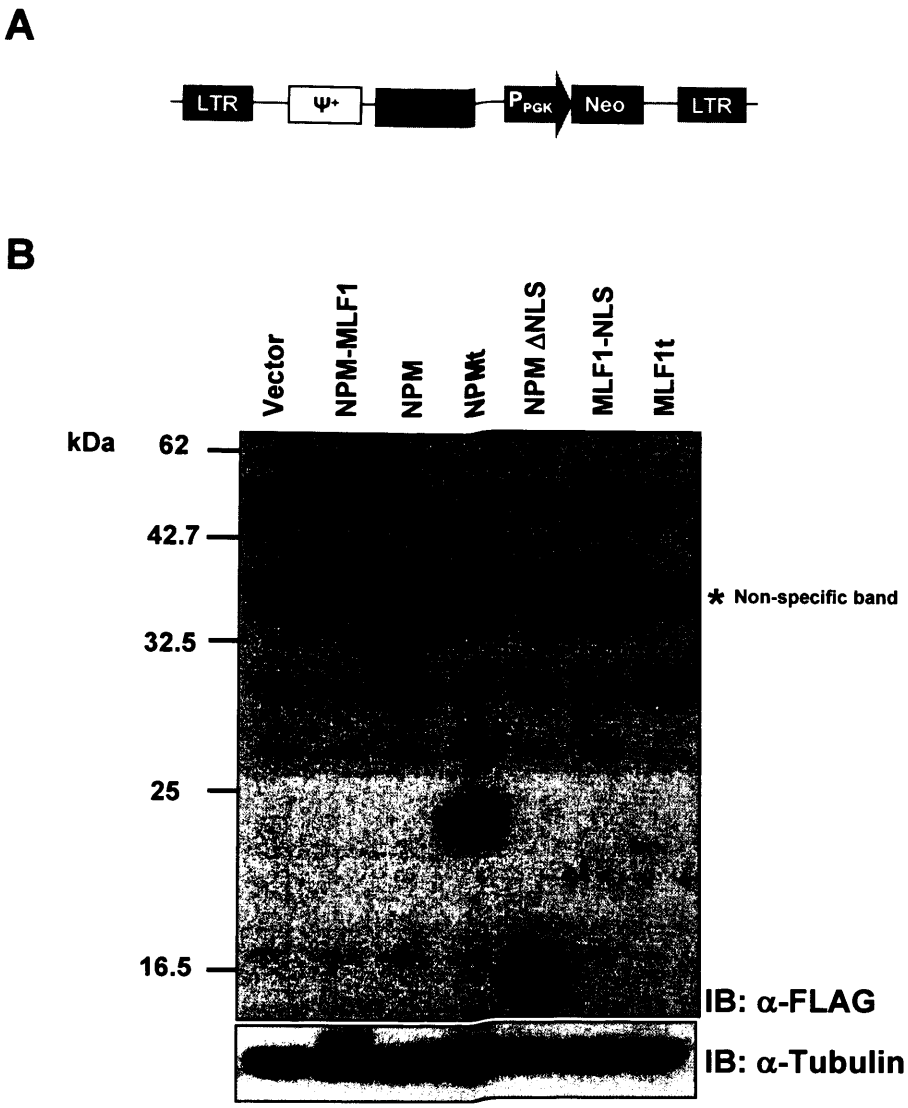


Figure 6.4. Expression of NPM and MLF1 deletion mutants. A. The PCR amplified *NPM* and *MLF1* fragments were cloned into pMSCV retroviral vector containing a neomycin resistance gene. **B.** 3T3 mouse fibroblasts were transduced with the deletion mutant retrovirus and were grown in G418 for 10 days. Total cell lysates were prepared and resolved on a 10% polyacrylamide gel. FLAG antibody was used to detect the proteins. An anti-tubulin antibody was used as loading control.

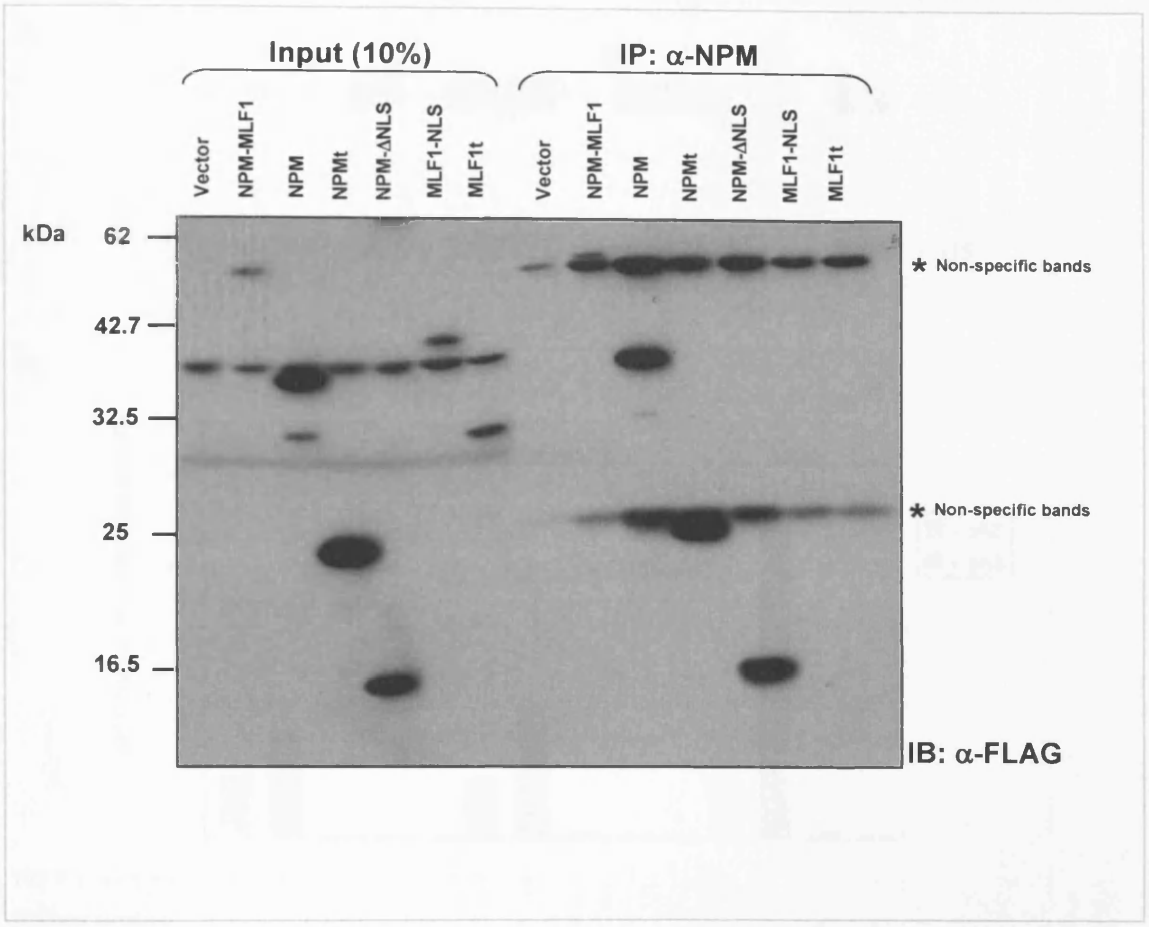


Figure 6.5. Endogenous NPM interacts with NPM moiety of NPM-MLF1. 3T3 cells were transduced with *tNPM*, *NPM ΔNLS*, *MLF1-NLS* and *tMLF1* retroviruses and were grown in selection for 10 days. As positive controls, cells were transduced with *NPM* and *NPM-MLF1* constructs. As a negative control, cells were transduced with empty vector retrovirus. 10% of total cell lysates were used as input. Immunoprecipitations were carried out using an NPM antibody and the FLAG antibody was used to identify FLAG-tagged protein in the immunoprecipitated complex. The proteins were resolved on a 10% polyacrylamide gel. Non-specific bands are shown by an asterisk.

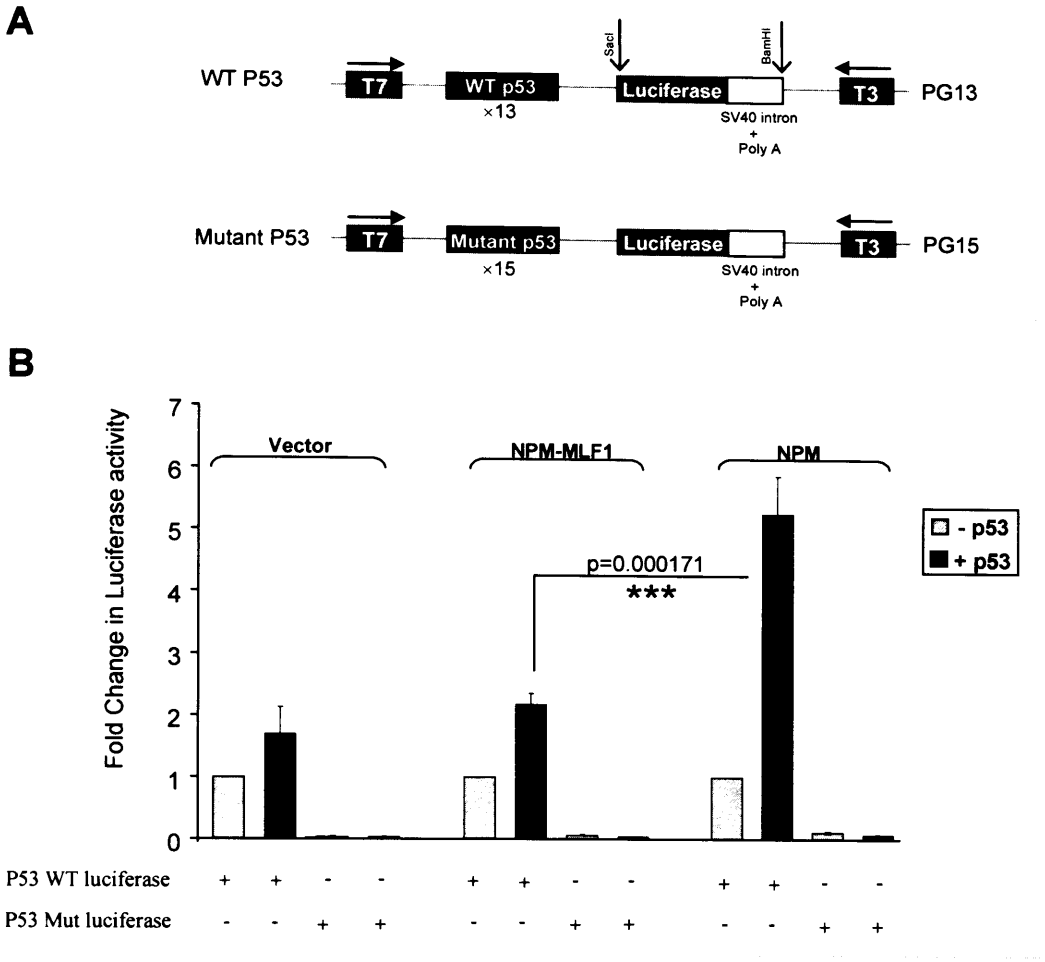


Figure 6.6. NPM activates p53 luciferase promoter activity. A. Wild type and mutant *p53* luciferase promoter used in transient transfection experiments. pG13 contains 13 copies of the p53-binding consensus sequence and pG15 contains 15 copies of a similar sequence mutated at critical positions (El-Deiry *et al* 1992). **B.** Mouse 3T3 fibroblasts were transiently transfected with either pCDNA3 expression vector alone (0.4 ug), *NPM-MLF1* (0.4 ug) or with *NPM* (0.4 ug). Cells were transfected with constant concentration of wild type p53-luciferase vector (0.05 ug) or with mutant *p53*-luciferase vector (0.05 ug). Cells were also transfected with (black bar) or without (grey bar) a p53 expression vector (0.2 ug). The p53-luciferase activity was measured 24 hr following transfection. The data represents mean and ± S.Ds of one experiment in triplicate. The NPM-mediated p53 activation is statistically significant p=0.000176) as indicated by asterisk.

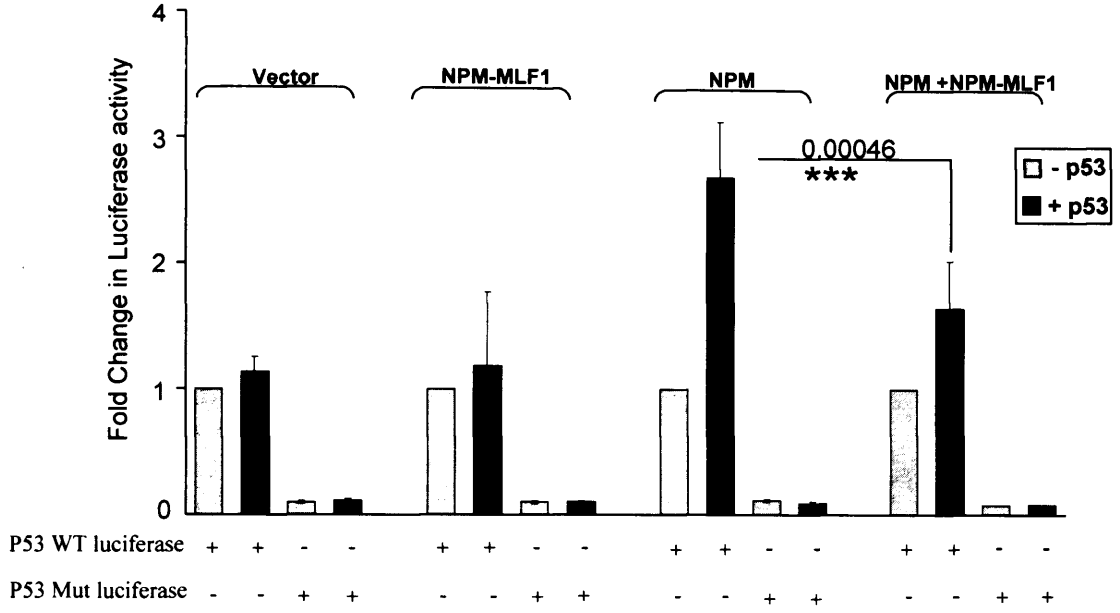


Figure 6.7. NPM-MLF1 inhibits NPM mediated p53 transcriptional activation. Mouse 3T3 fibroblasts were transiently transfected with either pCDNA3 expression vector alone (0.4 ug), *NPM-MLF1* (0.4 ug), *NPM* (0.4 ug), or co-tranfected with *NPM* and *NPM-MLF1*. Cells were transfected with a constant concentration of wild type p53-luciferase vector (0.05 ug) or with a mutant p53-luciferase vector (0.05 ug). The cells were also transfected with (black bar) or without (grey bar) a p53 expression vector (0.2 ug). The p53-luciferase activity was measured 24 hr following transfection. The data represents mean and ± standard S.Ds of one experiment in triplicate. The statistical significance of the NPM-MLF1 mediated repression is p=0.00046 (as indicated by asterisk).

6.7 Discussion

To gain more insight into the function of NPM-MLF1 and potentially the pathogenesis of MDS we aimed to examine the potential interaction between this fusion protein and wild type NPM. NPM is localised in the nucleoli and since NPM-MLF1 shares the same sub-cellular localisation (Yoneda-Kato et al., 1996) we set out to investigate whether these two proteins interact with each other and examined the functional significance of this interaction in the context of p53 activity. Immunoprecipitation revealed that NPM-MLF1 did interact with NPM in mouse fibroblasts. To establish the region required for this interaction, several deletion constructs were generated. The NPM moiety of the fusion protein was found to be essential for this interaction, probably mediated by the homodimerization domain at the N-terminus of the NPM protein.

A construct containing the MLF1 portion found in NPM-MLF1, joined to the NPM-derived NLS motif was generated. This NLS was thought to be sufficient for translocation of MLF1 into the nucleus, where the protein could interact with other nuclear proteins. We demonstrated that this region of the protein does not interact with endogenous NPM. MLF1 was thought to be a cytoplasmic protein which was retained in the cytoplasm by interaction with 14-3-3 proteins. Recent published data have identified two new functional domains of MLF, a nuclear export signal at residues 88-100, and two NLS, located at residues 168-174 and 232-236 (Winteringham et al., 2006; Yoneda-Kato and Kato, 2008). MLF1 was shown to also be localised to the nucleus. These data indicate that the MLF1 region of the fusion protein contains NLS and NES motifs, which questions the hypothesis that

NPM-MLF1 induces leukaemogenesis primarily as result of delocalising MLF1 to the nucleus.

We have shown in transient transfection studies, that NPM enhances p53-mediated activation of a p53 luciferase promoter. NPM directly interacts with p53 via residues 249-262 located at the C-terminus region (Lambert and Buckle, 2006). There are several lines of evidence to suggest that NPM increases the stability and hence transcriptional activity of the p53 tumour suppressor protein (Colombo et al., 2002; Kurki et al., 2004). One mechanism by which NPM increases the activity of p53 is by interacting with Mdm2 (Hdm2 in humans), repressing Mdm2-mediated p53 degradation through the proteosome (Kurki et al., 2004). In contrast, NPM has also been shown to negatively regulate p53 activity (Maiguel et al., 2004). Based on the current published data, it appears that the p53 regulation by NPM is dependant on several important factors such as the type of cell line used in the study, irradiation dose and the level of NPM expression in the cell.

The mechanism by which NPM affects p53 function is also dependent on the tight regulation of several cellular proteins and the physiological state of the cell. One of these important cellular components, involved in the regulation of growth and cell death, is the tumour suppressor ARF (Alternative Reading Frame-p19^{ARF}). This protein interacts with and controls the function of both NPM and p53 (**Figure 6.9A**). NPM and ARF are involved in a coregulatory loop, whereby, in a physiological state moderate levels of NPM maintain ARF in the nucleoli. This prevents ARF transport into the nucleoplasm, where it inhibits Mdm2-mediated p53 degradation and induces growth arrest or apoptosis (Bertwistle et al., 2004;

Korgaonkar et al., 2005). This interaction between NPM and ARF also prevents ARF degradation, by retaining the protein in the nucleolus. Upon oncogenic stress, however, expression of both ARF and NPM are upregulated. As ARF expression is increased, it translocates to the nucleoplasm, where it induces programmed cell death by inhibiting Mdm2 degradation of p53. As ARF expression becomes stabilised in the cell, it represses NPM-rRNA processing, which is required for cellular growth, by promoting ubiquitin dependent degradation of NPM. However, upregulation of NPM, and other genetic mutations can override the negative regulation of ARF and result in uncontrolled cellular proliferation.

We have shown that in contrast to NPM, NPM-MLF1 does not affect p53 activity in mouse fibroblasts. We therefore examined whether NPM-MLF1 can enhance or have a dominant negative effect on NPM function. Co-transfection experiments revealed that co-expression of NPM with NPM-MLF1 resulted in a significant repression of p53 promoter activation by NPM. Neither the p53-binding region nor the region required for the interaction with ARF, are present in NPM-MLF1. ARF is an important regulatory component of NPM-Mdm2-p53 pathway. For this reason, in future experiments, we would like to determine whether ARF is in the NPM-MLF1-NPM complex and examine the effect of these interactions on ARF activity.

A recent study has found that NPM causes a reduction in the levels of p53 protein in BM-derived HPCs (Li et al., 2006). In addition, *in vivo* data have revealed the importance of NPM in suppression of p53 function. $NPM^{-/-}$ MEFs were shown to be more susceptible to p53-mediated cell death than $NPM^{+/-}$ cells (Grisendi et al., 2005). It is believed that NPM can act as an oncogene or a tumour suppressor

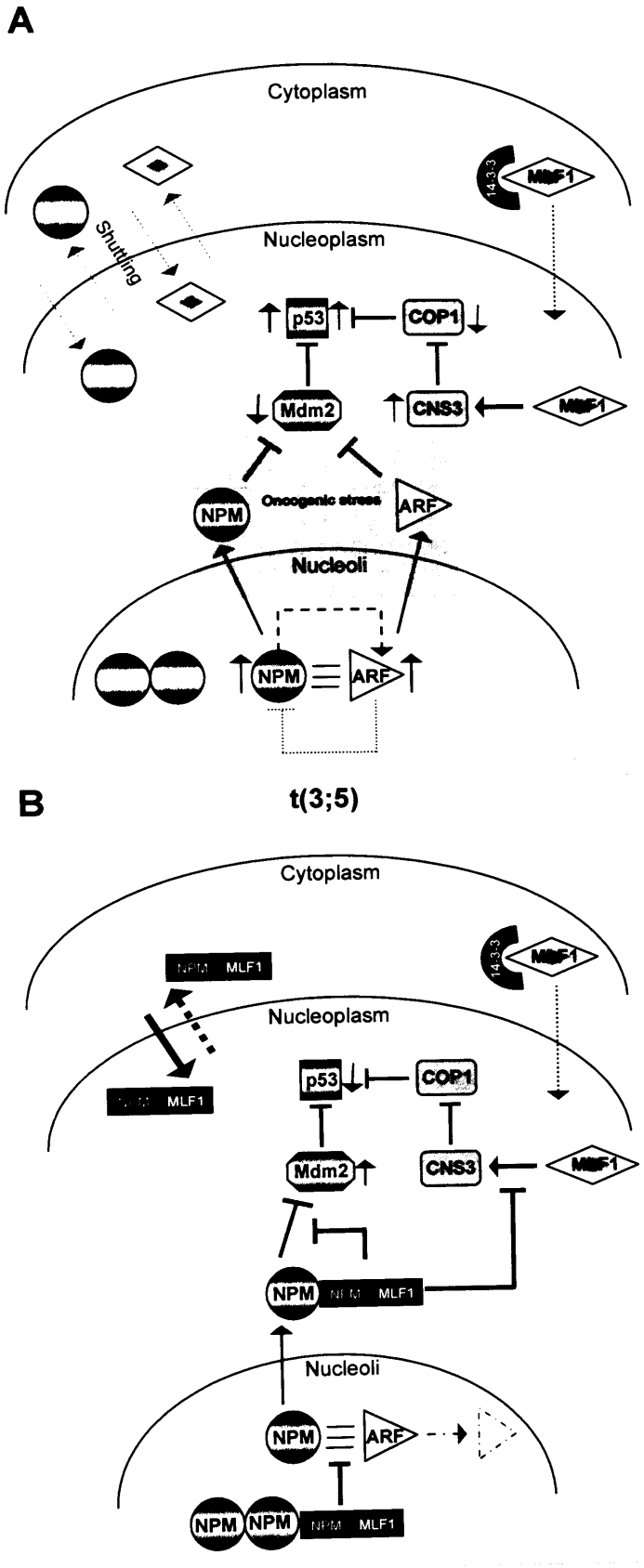
gene depending on its level of expression. It would be interesting to determine if NPM-MLF1 can rescue the *NPM*$^{-/-}$ cells from senescence or whether it would repress the function of NPM and induce premature senescence, comparable to what is detected in *NPM*$^{-/-}$ MEFs.

In the context of MDS and progression to AML, cells harbouring t(3;5) may be more susceptible to ARF degradation. These cells have low levels of NPM due to one NPM allele being disrupted by a translocation. This may result in reduced NPM binding by ARF, and hence increased ARF degradation (**Figure 6.9B**). Furthermore, NPM-MLF1 may compete with ARF for NPM binding in the nucleoli. This would also be expected to result in increased ARF degradation. The net effect of increased ARF degradation, in cells harbouring NPM-MLF1, is increased Mdm2 mediated p53 degradation, causing unregulated proliferation of cells, which in the presence of additional genetic mutations could lead to leukaemic progression. It is possible that NPM-MLF1 may also induce leukaemogenesis by regulating the function of both NPM and MLF1, inhibiting the Mdm2-p53 and CNS3-COP1-53 tumour suppressor pathway, respectively. Evidence showing that NPM-MLF1 reduces p53 expression upon oncogenic stress provides support for this hypothesis (Yoneda-Kato and Kato, 2008).

Furthermore, in a normal state, the shuttling of MLF1 between the nucleus and cytoplasm is tightly regulated and in MDS, the shuttling imbalance of MLF1 due to t(3;5) can lead to leukaemogenesis. This hypothesis is supported by the observation that the MLF1-derived NES motif is required for the function of wild type MLF1 and NPM-MLF1. Expression of MLF1 harbouring a mutation in the NES domain

resulted in enhanced p53 activity in mouse fibroblasts, as compared with cells expressing wild type MLF1. This suggests that the shuttling activity of MLF1 is essential for regulation of p53 function. Furthermore, cellular transformation of MEFs and increased proliferation of BM-derived HPCs induced by NPM-MLF1 was abrogated by disrupting the NES sequence in the MLF1 moiety of the fusion protein (Yoneda-Kato and Kato, 2008). The level of p53 and p21 was reduced in NPM-MLF1 expressing cells than in control cells and treatment with UV irradiation failed to upregulate p53 and p21 upregulation in these cells but there was an accumulation of p53 and p21 in cells expressing NPM-MLF1 containing a mutation in MLF1 derived NES motif. This study suggests that increased shuttling activity of NPM-MLF1 may have an important role in cellular proliferation in MDS patients. In accordance with this, mutations in NPM that lead to the acquisition of an NES cause abnormal localization of the protein in the cytoplasm (NPMc+). These mutations are highly associated with AML patients and further highlight the importance of shuttling imbalances in leukaemogenesis. Our results show that NPM-MLF1 interacts with NPM and affects the NPM-mediated p53 regulation. Understanding the mechanism by which NPM-MLF1 influences the p53 activity would allow a better understanding of the pathogenesis of MDS.

Figure 6.9. Regulation of the p53 tumour suppressor pathway. A. The regulation of ARF-Mdm2-p53 by NPM and MLF1. NPM and ARF exist in a negative regulatory loop, whereby NPM forms a complex with ARF preventing it from degradation as well as retaining the protein in the nucleoli. ARF regulates NPM subcellular shuttling activity through its direct interaction with the protein, thus retaining it in the nucleoli. In addition, ARF inhibits NPM ribosome biogenesis by inducing NPM polyubiquitination and proteosome degradation. Upon oncogenic stress, NPM expression is increased, which results in the stabilisation of ARF. This leads to ARF translocation into the nucleoplasm where it induces growth arrest by inhibiting Mdm2- mediated p53 degradation. NPM also positively regulates p53 activation by suppressing Mdm2 activity. NPM can homodimerise and it can shuttle between the cytoplasm and the nucleoli where it is predominantly localised. In contrast MLF1 is mainly present in the cytoplasm through interaction with 14-3-3 proteins, but it can shuttle between the cytoplasm and nucleus. MLF1 also induces cell cycle arrest by inhibiting CNS3-COP1 mediated degradation of p53. **B. The role of NPM-MF1 in regulating the tumour suppressor pathway in MDS.** NPM-MLF1, formed as a result of t(3;5), is localised in the nucleoli where it can interact with the remaining NPM protein pool. NPM-MF1 may compete with ARF for interaction with NPM, which can ultimately result in ARF degradation. Alternatively, NPM-MLF1 interaction with NPM may suppress NPM mediated activation of p53. There is also increased sub-cellular shuttling of NPM-MLF1 protein due to the acquisition of NLS and NES motifs derived from the MLF1 moiety of NPM-MLF. The loss of one MLF1 allele due to t(3;5) may cause aberrant regulation of the CNS3-COP1-p53 pathway. Alternatively, NPM-MLF1 may inhibit p53 mediated cell cycle arrest by repressing the function of wild type MLF1. The net affect of these events would be uncontrolled cellular proliferation.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Cytoplasm
14-3-3
MLF1
Shuttling
Nucleoplasm
p53
COP1
Mdm2
CNS3
MLF1
NPM
Oncogenic stress
ARF
Nucleoli
NPM
ARF
B
t(3;5)
Cytoplasm
14-3-3
MLF1
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Nucleoplasm
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COP1
Mdm2
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7.0 Conclusions

The main aim of this project was to investigate the molecular basis of AMKL and MDS. DS-AMKL is an ideal model for studying AMKL as the molecular predisposition is known in these individuals, thus allowing us to identify genes and pathways that lead to AMKL development. DS patients are highly predisposed to AMKL development as compared to non-DS patients. Furthermore, AMKL patients with DS, but not those without DS, have somatic mutations in the X-linked gene, *GATA1*. The extra copy of genes on chromosome 21 is another predisposition factor in DS patients that is believed to be important in AMKL development. A number of studies have attributed the increased sensitivity of DS-AMKL to therapy, to overexpression of chromosome 21 genes. Defining a part of the molecular pathway leading to AMKL would not only lead to more specific therapies for DS-AMKL but may also yield important mechanistic insights into AMKL in general.

The potential link between a chromosome 21 gene, *ERG-3,* and the *GATA1s* mutation was investigated in murine haematopoiesis. This study indicates that *ERG-3* has a role in megakaryopoiesis and it collaborates with the *GATA1s* mutation to immortalize progenitor cells *in vitro*, and induce leukaemia *in vivo*, with an early megakaryoblastic phenotype. ERG-3 was shown to be oncogenic *in vivo* and it immortalized foetal liver derived progenitors only in the presence of specific cytokines.

To further understand the development of AMKL, future studies should focus on isolating putative leukaemic stem cells responsible for the initiation and

maintenance of the disease. In addition, identifying GATA1 target genes that are dysregulated by GATA1s and ERG-3 would identify molecular pathways that are involved in AMKL. This study highlights the pro-leukaemic effect of the overexpression of a chromosome 21 gene, as seen in DS-AMKL individuals, and its collaborative effect with the GATA1s mutation in enhancing the proliferation of haematopoietic progenitor cells. We propose that ERG-3 promotes megakaryopoesis and synergises with GATA1s to increase the proliferation of megakaryoblasts. In addition, the deregulation of genes encoding tryosine kinases (KIT/JAK2/FLT3), or genes in the RAS pathway (NRAS/KRAS), may enhance the proliferation or survival of stem cells leading to AMKL development in DS patients.

The molecular basis of MDS was also investigated in this study. Peripheral blood cytopenia, high levels of apoptosis in the bone marrow, abnormal development and differentiation of cells of the myeloid lineage and frequent progression to AML are all hallmarks of MDS. Based on the colony forming assays carried out in this study, it appears that the role of NPM-MLF1 in the pathogenesis of MDS may be cell context dependent. In myeloid cells, the fusion protein impairs myeloid differentiation whereas it does not affect the development of megakaryocytes. The transient expansion of progenitor cells may reflect the clonal expansion detected in MDS patients and other factors may be required for cooperation with the fusion protein for transformation of progenitor cells *in vitro* and progression into AML. Megakaryocyte development was demonstrated to be enhanced by NPM, but not NPM-MLF1. It is possible that the effect of NPM-MLF1 is masked by the endogenous NPM in haematopoietic progenitors. The megakaryodysplasia detected in MDS individuals may be as a result of reduced expression levels of NPM, since

one NPM allele is involved in chromosomal translocation. In addition, NPM-MLF1 was shown to interact with its wild type counterpart NPM. This interaction resulted in inhibition of NPM-mediated p53 activation in mouse fibroblasts. Both p53 and ARF have been shown to regulate cellular proliferation through induction of growth arrest or apoptosis. Reduced expression levels and a shuttling imbalance of NPM and MLF1 can lead to reduced activation of both p53 and ARF, which result in increased cellular proliferation. The increased cellular proliferation by NPM-MLF1 may be mediated by the impaired shuttling activity of the protein, which is induced by the acquisition of NLS and NES sequences, from its MLF1 and NPM moiety. These events, in combination with acquisition of mutations in genes required for cell maintenance and DNA repair as well as increased expression levels of anti-apoptotic molecules, could result in MDS progression to AML.

A major goal in developing novel and specific therapies for MDS is the induction of apoptosis and/or differentiation of the aberrant haematopoietic progenitor cells causing the disease. Future studies should focus on identifying the target cell of transformation in MDS and factors that contribute to the progression of MDS to AML. It is also important to use the fusion gene to develop a mouse model for MDS that recapitulates all the features of human disease. Transplantation assays using bone marrow derived progenitors expressing NPM-MLF1 may provide useful insight to the role of the fusion protein in the multi-step pathogenesis of MDS. Furthermore, the use of $NPM^{+/-}$ mice would be an ideal model to dissect the effect of NPM-MLF1 on HSC and progenitor cell development. The $NPM^{+/-}$ progenitor cells resemble progenitor cells found in MDS patients as they were shown to be defective in megakaryocyte and erythrocyte differentiation.

In conclusion, this study provides support for the multi-step nature of leukaemogenesis. In the DS-AMKL model, the mechanism of leukaemogenesis appears to be based on the underlying GATA1s mutation associated with the disease in a context of a specific genetic background, which is trisomy 21. Loss of full GATA1 protein provides a loss of differentiation signal, while acquisition of GATA1s provides the pro-proliferation signal required for initiation of leukaemic transformation. In the MDS model, acquisition of t(3;5) most likely represents an early event in the multi-step development of MDS. The NPM-MLF1 fusion protein inhibits differentiation of myeloid cells and can deregulate the p53 pathway required for DNA repair and proliferation either directly, or via interference with the function of its wild type counterparts NPM and / MLF1. Analysing the potential transforming and oncogenic activities of ERG-3 and GATA1s, in AMKL, and NPM-MLF1 in MDS will yield important mechanistic insights into these diseases.

8.0 References

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